

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
15 September 1994 (15.09.1994)

PCT

(10) International Publication Number  
WO 94/020127 A1

- (51) International Patent Classification<sup>5</sup>: A61K 37/02, C07K 7/06
- (74) Agents: BASTIAN, Kevin, L. et al.; Townsend and Townsend Kourie and Crew, Steuart Street Tower, 20th floor, One Market Plaza, San Francisco, CA 94105 (US).
- (21) International Application Number: PCT/US94/02353
- (81) Designated States (*national*): AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, UA, UZ, VN.
- (22) International Filing Date: 4 March 1994 (04.03.1994)
- (25) Filing Language: English
- (84) Designated States (*regional*): European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
- (26) Publication Language: English
- (30) Priority Data:  
08/027,146 5 March 1993 (05.03.1993) US  
08/073,205 4 June 1993 (04.06.1993) US  
08/159,184 29 November 1993 (29.11.1993) US
- Published:  
— with international search report
- (71) Applicant: CYTEL CORPORATION [US/US]; 3525 John Hopkins Court, San Diego, CA 92121 (US).
- (48) Date of publication of this corrected version: 17 April 2003
- (72) Inventors: GREY, Howard, M.; 9066 La Jolla Shores Lane, La Jolla, CA 92037 (US). SETTE, Alessandro; 5551 Linda Rosa Avenue, La Jolla, CA 92037 (US). SIDNEY, John; 8541 D Villa La Jolla Drive, La Jolla, CA 92037 (US). KAST, W., Martin; Maria Rutgersweg 106, NL-2331 NX Leiden (NL).
- (15) Information about Correction:  
see PCT Gazette No. 16/2003 of 17 April 2003, Section II
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*



WO 94/020127 A1

(54) Title: HLA-A2.1 BINDING PEPTIDES AND THEIR USES

(57) Abstract: The present invention provides the means and methods for selecting immunogenic peptides and the immunogenic peptide compositions capable of specifically binding glycoproteins encoded by HLA-A2.1 allele and inducing T cell activation in T cells restricted by the A2.1 allele. The peptides are useful to elicit an immune response against a desired antigen.

AA

USSN: 09/854,248

Atty. Docket No.:

020093-000810US

BEST AVAILABLE COPY

### HLA-A2.1 BINDING PEPTIDES AND THEIR USES

5           The present application is a continuation in part of  
USSN 08/159,184, which is a continuation in part of USSN  
08/073,205, which is a continuation in part of USSN  
08/027,146, all of which are incorporated herein by reference.

### BACKGROUND OF THE INVENTION

10           The present invention relates to compositions and  
methods for preventing, treating or diagnosing a number of  
pathological states such as viral diseases and cancers. In  
15       particular, it provides novel peptides capable of binding  
selected major histocompatibility complex (MHC) molecules and  
inducing an immune response.

          MHC molecules are classified as either Class I or  
Class II molecules. Class II MHC molecules are expressed  
20       primarily on cells involved in initiating and sustaining  
immune responses, such as T lymphocytes, B lymphocytes,  
macrophages, etc. Class II MHC molecules are recognized by  
helper T lymphocytes and induce proliferation of helper T  
lymphocytes and amplification of the immune response to the  
25       particular immunogenic peptide that is displayed. Class I MHC  
molecules are expressed on almost all nucleated cells and are  
recognized by cytotoxic T lymphocytes (CTLs), which then  
destroy the antigen-bearing cells. CTLs are particularly  
important in tumor rejection and in fighting viral infections.

30       The CTL recognizes the antigen in the form of a  
peptide fragment bound to the MHC class I molecules rather  
than the intact foreign antigen itself. The antigen must  
normally be endogenously synthesized by the cell, and a  
portion of the protein antigen is degraded into small peptide  
35       fragments in the cytoplasm. Some of these small peptides  
translocate into a pre-Golgi compartment and interact with  
class I heavy chains to facilitate proper folding and  
association with the subunit  $\beta$ 2 microglobulin. The

peptide-MHC class I complex is then routed to the cell surface for expression and potential recognition by specific CTLs.

Investigations of the crystal structure of the human MHC class I molecule, HLA-A2.1, indicate that a peptide binding groove is created by the folding of the  $\alpha 1$  and  $\alpha 2$  domains of the class I heavy chain (Bjorkman et al., Nature 329:506 (1987)). In these investigations, however, the identity of peptides bound to the groove was not determined.

Buus et al., Science 242:1065 (1988) first described a method for acid elution of bound peptides from MHC. Subsequently, Rammensee and his coworkers (Falk et al., Nature 351:290 (1991) have developed an approach to characterize naturally processed peptides bound to class I molecules. Other investigators have successfully achieved direct amino acid sequencing of the more abundant peptides in various HPLC fractions by conventional automated sequencing of peptides eluted from class I molecules of the B type (Jardetzky, et al., Nature 353:326 (1991) and of the A2.1 type by mass spectrometry (Hunt, et al., Science 225:1261 (1992)). A review of the characterization of naturally processed peptides in MHC Class I has been presented by Rötzschke and Falk (Rötzschke and Falk, Immunol. Today 12:447 (1991)).

Sette et al., Proc. Natl. Acad. Sci. USA 86:3296 (1989) showed that MHC allele specific motifs could be used to predict MHC binding capacity. Schaeffer et al., Proc. Natl. Acad. Sci. USA 86:4649 (1989) showed that MHC binding was related to immunogenicity. Several authors (De Bruijn et al., Eur. J. Immunol., 21:2963-2970 (1991); Pamer et al., 991 Nature 353:852-955 (1991)) have provided preliminary evidence that class I binding motifs can be applied to the identification of potential immunogenic peptides in animal models. Class I motifs specific for a number of human alleles of a given class I isotype have yet to be described. It is desirable that the combined frequencies of these different alleles should be high enough to cover a large fraction or perhaps the majority of the human outbred population.

Despite the developments in the art, the prior art has yet to provide a useful human peptide-based vaccine or

therapeutic agent based on this work. The present invention provides these and other advantages.

#### SUMMARY OF THE INVENTION

5           The present invention provides compositions comprising immunogenic peptides having binding motifs for HLA-A2.1 molecules. The immunogenic peptides, which bind to the appropriate MHC allele, are preferably 9 to 10 residues in length and comprise conserved residues at certain positions  
10       such as positions 2 and 9. Moreover, the peptides do not comprise negative binding residues as defined herein at other positions such as positions 1, 3, 6 and/or 7 in the case of peptides 9 amino acids in length and positions 1, 3, 4, 5, 7, 8 and/or 9 in the case of peptides 10 amino acids in length.  
15       The present invention defines positions within a motif enabling the selection of peptides which will bind efficiently to HLA A2.1.

          Epitopes on a number of immunogenic target proteins can be identified using the peptides of the invention.  
20       Examples of suitable antigens include prostate cancer specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, human immunodeficiency type-1 virus (HIV1) and papilloma virus antigens. The peptides are thus useful in pharmaceutical  
25       compositions for both in vivo and ex vivo therapeutic and diagnostic applications.

#### Definitions

          The term "peptide" is used interchangeably with  
30       "oligopeptide" in the present specification to designate a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of adjacent amino acids. The oligopeptides of the invention are less than about 15 residues  
35       in length and usually consist of between about 8 and about 11 residues, preferably 9 or 10 residues.

          An "immunogenic peptide" is a peptide which comprises an allele-specific motif such that the peptide will



bind an MHC molecule and induce a CTL response. Immunogenic peptides of the invention are capable of binding to an appropriate HLA-A2.1 molecule and inducing a cytotoxic T cell response against the antigen from which the immunogenic peptide is derived.

Immunogenic peptides are conveniently identified using the algorithms of the invention. The algorithms are mathematical procedures that produce a score which enables the selection of immunogenic peptides. Typically one uses the algorithmic score with a "binding threshold" to enable selection of peptides that have a high probability of binding at a certain affinity and will in turn be immunogenic. The algorithm is based upon either the effects on MHC binding of a particular amino acid at a particular position of a peptide or the effects on binding of a particular substitution in a motif containing peptide.

A "conserved residue" is an amino acid which occurs in a significantly higher frequency than would be expected by random distribution at a particular position in a peptide. Typically a conserved residue is one where the MHC structure may provide a contact point with the immunogenic peptide. One to three, preferably two, conserved residues within a peptide of defined length defines a motif for an immunogenic peptide. These residues are typically in close contact with the peptide binding groove, with their side chains buried in specific pockets of the groove itself. Typically, an immunogenic peptide will comprise up to three conserved residues, more usually two conserved residues.

As used herein, "negative binding residues" are amino acids which if present at certain positions (for example, positions 1, 3 and/or 7 of a 9-mer) will result in a peptide being a nonbinder or poor binder and in turn fail to be immunogenic i.e. induce a CTL response.

The term "motif" refers to the pattern of residues in a peptide of defined length, usually about 8 to about 11 amino acids, which is recognized by a particular MHC allele. The peptide motifs are typically different for each human MHC

allele and differ in the pattern of the highly conserved residues and negative residues.

The binding motif for an allele can be defined with increasing degrees of precision. In one case, all of the conserved residues are present in the correct positions in a peptide and there are no negative residues in positions 1,3 and/or 7.

The phrases "isolated" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state. Thus, the peptides of this invention do not contain materials normally associated with their in situ environment, e.g., MHC I molecules on antigen presenting cells. Even where a protein has been isolated to a homogenous or dominant band, there are trace contaminants in the range of 5-10% of native protein which co-purify with the desired protein. Isolated peptides of this invention do not contain such endogenous co-purified protein.

The term "residue" refers to an amino acid or amino acid mimetic incorporated in an oligopeptide by an amide bond or amide bond mimetic.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram of an HLA-A purification scheme.

Fig. 2 shows a scattergram of the log of relative binding plotted against the "Grouped Ratio" algorithm for 9 mer peptides.

Fig. 3 shows a scattergram of the log of relative binding plotted against the average "Log of Binding" algorithm score for 9 mer peptides.

Figs. 4 and 5 show scattergrams of a set of 10-mer peptides containing preferred residues in positions 2 and 10 as scored by the "Grouped Ratio" and "Log of Binding" algorithms.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the determination of allele-specific peptide motifs for human Class I MHC (sometimes referred to as HLA) allele subtypes, in particular, peptide motifs recognized by HLA-A2.1 alleles. These motifs are then used to define T cell epitopes from any desired antigen, particularly those associated with human viral diseases, cancers or autoimmune diseases, for which the amino acid sequence of the potential antigen or autoantigen targets is known.

Epitopes on a number of potential target proteins can be identified in this manner. Examples of suitable antigens include prostate specific antigen (PSA), hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, melanoma antigens (e.g., MAGE-1), human immunodeficiency virus (HIV) antigens and human papilloma virus (HPV) antigens.

The peptides of the invention may also be employed to relieve the symptoms of, treat or prevent the occurrence or reoccurrence of autoimmune diseases. Such diseases include, for example, multiple sclerosis (MS), rheumatoid arthritis (RA), Sjogren syndrome, scleroderma, polymyositis, dermatomyositis, systemic lupus erythematosus, juvenile rheumatoid arthritis, ankylosing spondylitis, myasthenia gravis (MG), bullous pemphigoid (antibodies to basement membrane at dermal-epidermal junction), pemphigus (antibodies to mucopolysaccharide protein complex or intracellular cement substance), glomerulonephritis (antibodies to glomerular basement membrane), Goodpasture's syndrome, autoimmune hemolytic anemia (antibodies to erythrocytes), Hashimoto's disease (antibodies to thyroid), pernicious anemia (antibodies to intrinsic factor), idiopathic thrombocytopenic purpura (antibodies to platelets), Grave's disease, and Addison's disease (antibodies to thyroglobulin), and the like.

The autoantigens associated with a number of these diseases have been identified. For example, in experimentally induced autoimmune diseases, antigens involved in pathogenesis have been characterized: in arthritis in rat and mouse,

native type-II collagen is identified in collagen-induced arthritis, and mycobacterial heat shock protein in adjuvant arthritis; thyroglobulin has been identified in experimental allergic thyroiditis (EAT) in mouse; acetyl choline receptor (AChR) in experimental allergic myasthenia gravis (EAMG); and myelin basic protein (MBP) and proteolipid protein (PLP) in experimental allergic encephalomyelitis (EAE) in mouse and rat. In addition, target antigens have been identified in humans: type-II collagen in human rheumatoid arthritis; and acetyl choline receptor in myasthenia gravis.

Peptides comprising the epitopes from these antigens are synthesized and then tested for their ability to bind to the appropriate MHC molecules in assays using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorometry, peptide-dependent class I assembly assays, and inhibition of CTL recognition by peptide competition. Those peptides that bind to the class I molecule are further evaluated for their ability to serve as targets for CTLs derived from infected or immunized individuals, as well as for their capacity to induce primary in vitro or in vivo CTL responses that can give rise to CTL populations capable of reacting with virally infected target cells or tumor cells as potential therapeutic agents.

The MHC class I antigens are encoded by the HLA-A, B, and C loci. HLA-A and B antigens are expressed at the cell surface at approximately equal densities, whereas the expression of HLA-C is significantly lower (perhaps as much as 10-fold lower). Each of these loci have a number of alleles. The peptide binding motifs of the invention are relatively specific for each allelic subtype.

For peptide-based vaccines, the peptides of the present invention preferably comprise a motif recognized by an MHC I molecule having a wide distribution in the human population. Since the MHC alleles occur at different frequencies within different ethnic groups and races, the choice of target MHC allele may depend upon the target population. Table 1 shows the frequency of various alleles at

the HLA-A locus products among different races. For instance, the majority of the Caucasoid population can be covered by peptides which bind to four HLA-A allele subtypes, specifically HLA-A2.1, A1, A3.2, and A24.1. Similarly, the majority of the Asian population is encompassed with the addition of peptides binding to a fifth allele HLA-A11.2.

TABLE 1

<u>A Allele/Subtype</u>	<u>N(69)*</u>	<u>A(54)</u>	<u>C(502)</u>
A1	10.1 (7)	1.8 (1)	27.4 (138)
A2.1	11.5 (8)	37.0 (20)	39.8 (199)
A2.2	10.1 (7)	0	3.3 (17)
A2.3	1.4 (1)	5.5 (3)	0.8 (4)
A2.4	-	-	-
A2.5	-	-	-
A3.1	1.4 (1)	0	0.2 (0)
A3.2	5.7 (4)	5.5 (3)	21.5 (108)
A11.1	0	5.5 (3)	0
A11.2	5.7 (4)	31.4 (17)	8.7 (44)
A11.3	0	3.7 (2)	0
A23	4.3 (3)	-	3.9 (20)
A24	2.9 (2)	27.7 (15)	15.3 (77)
A24.2	-	-	-
A24.3	-	-	-
A25	1.4 (1)	-	6.9 (35)
A26.1	4.3 (3)	9.2 (5)	5.9 (30)
A26.2	7.2 (5)	-	1.0 (5)
A26V	-	3.7 (2)	-
A28.1	10.1 (7)	-	1.6 (8)
A28.2	1.4 (1)	-	7.5 (38)
A29.1	1.4 (1)	-	1.4 (7)
A29.2	10.1 (7)	1.8 (1)	5.3 (27)
A30.1	8.6 (6)	-	4.9 (25)
A30.2	1.4 (1)	-	0.2 (1)
A30.3	7.2 (5)	-	3.9 (20)
A31	4.3 (3)	7.4 (4)	6.9 (35)
A32	2.8 (2)	-	7.1 (36)
Aw33.1	8.6 (6)	-	2.5 (13)
Aw33.2	2.8 (2)	16.6 (9)	1.2 (6)
Aw34.1	1.4 (1)	-	-
Aw34.2	14.5 (10)	-	0.8 (4)
Aw36	5.9 (4)	-	-

Table compiled from B. DuPont, Immunobiology of HLA, Vol. I, Histocompatibility Testing 1987, Springer-Verlag, New York 1989.

\* N - negroid; A = Asian; C = caucasoid. Numbers in parenthesis represent the number of individuals included in the analysis.

The nomenclature used to describe peptide compounds follows the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue. In the formulae representing selected specific embodiments of the present invention, the amino- and carboxyl-terminal groups, although not specifically shown, are in the form they would assume at physiologic pH values, unless otherwise specified. In the amino acid structure formulae, each residue is generally represented by standard three letter or single letter designations. The L-form of an amino acid residue is represented by a capital single letter or a capital first letter of a three-letter symbol, and the D-form for those amino acids having D-forms is represented by a lower case single letter or a lower case three letter symbol. Glycine has no asymmetric carbon atom and is simply referred to as "Gly" or G.

The procedures used to identify peptides of the present invention generally follow the methods disclosed in Falk et al., Nature 351:290 (1991), which is incorporated herein by reference. Briefly, the methods involve large-scale isolation of MHC class I molecules, typically by immunoprecipitation or affinity chromatography, from the appropriate cell or cell line. Examples of other methods for isolation of the desired MHC molecule equally well known to the artisan include ion exchange chromatography, lectin chromatography, size exclusion, high performance ligand chromatography, and a combination of all of the above techniques.

In the typical case, immunoprecipitation is used to isolate the desired allele. A number of protocols can be used, depending upon the specificity of the antibodies used. For example, allele-specific mAb reagents can be used for the affinity purification of the HLA-A, HLA-B<sub>1</sub>, and HLA-C molecules. Several mAb reagents for the isolation of HLA-A molecules are available. The monoclonal BB7.2 is suitable for isolating HLA-A2 molecules. Affinity columns prepared with

these mAbs using standard techniques are successfully used to purify the respective HLA-A allele products.

In addition to allele-specific mAbs, broadly reactive anti-HLA-A, B, C mAbs, such as W6/32 and B9.12.1, and one anti-HLA-B, C mAb, B1.23.2, could be used in alternative affinity purification protocols as described in the example section below.

The peptides bound to the peptide binding groove of the isolated MHC molecules are eluted typically using acid treatment. Peptides can also be dissociated from class I molecules by a variety of standard denaturing means, such as heat, pH, detergents, salts, chaotropic agents, or a combination thereof.

Peptide fractions are further separated from the MHC molecules by reversed-phase high performance liquid chromatography (HPLC) and sequenced. Peptides can be separated by a variety of other standard means well known to the artisan, including filtration, ultrafiltration, electrophoresis, size chromatography, precipitation with specific antibodies, ion exchange chromatography, isoelectrofocusing, and the like.

Sequencing of the isolated peptides can be performed according to standard techniques such as Edman degradation (Hunkapiller, M.W., et al., Methods Enzymol. 91, 399 [1983]). Other methods suitable for sequencing include mass spectrometry sequencing of individual peptides as previously described (Hunt, et al., Science 225:1261 (1992), which is incorporated herein by reference). Amino acid sequencing of bulk heterogenous peptides (e.g., pooled HPLC fractions) from different class I molecules typically reveals a characteristic sequence motif for each class I allele.

Definition of motifs specific for different class I alleles allows the identification of potential peptide epitopes from an antigenic protein whose amino acid sequence is known. Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of motifs. The epitopic sequences are then synthesized. The



capacity to bind MHC Class molecules is measured in a variety of different ways. One means is a Class I molecule binding assay as described in Example 4, below. Other alternatives described in the literature include inhibition of antigen  
5 presentation (Sette, et al., J. Immunol. 141:3893 (1991), in vitro assembly assays (Townsend, et al., Cell 62:285 (1990), and FACS based assays using mutated cells, such as RMA.S (Melief, et al., Eur. J. Immunol. 21:2963 (1991)).

Next, peptides that test positive in the MHC class I  
10 binding assay are assayed for the ability of the peptides to induce specific CTL responses in vitro. For instance, Antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. Antigen-presenting cells can  
15 be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., J. Exp. Med. 166:182 (1987); Boog, Eur. J. Immunol. 18:219 [1988]).

Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with  
20 internally processed peptides, such as the mouse cell lines RMA-S (Kärre, et al., Nature, 319:675 (1986); Ljunggren, et al., Eur. J. Immunol. 21:2963-2970 (1991)), and the human somatic T cell hybrid, T-2 (Cerundolo, et al., Nature 345:449-452 (1990)) and which have been transfected with the  
25 appropriate human class I genes are conveniently used, when peptide is added to them, to test for the capacity of the peptide to induce in vitro primary CTL responses. Other eukaryotic cell lines which could be used include various insect cell lines such as mosquito larvae (ATCC cell lines CCL  
30 125, 126, 1660, 1591, 6585, 6586), silkworm (ATCC CRL 8851), armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila cell lines such as a Schneider cell line (see Schneider J. Embryol. Exp. Morphol. 27:353-365 [1927]).

Peripheral blood lymphocytes are conveniently  
35 isolated following simple venipuncture or leukapheresis of normal donors or patients and used as the responder cell sources of CTL precursors. In one embodiment, the appropriate antigen-presenting cells are incubated with 10-100  $\mu$ M of

peptide in serum-free media for 4 hours under appropriate culture conditions. The peptide-loaded antigen-presenting cells are then incubated with the responder cell populations in vitro for 7 to 10 days under optimized culture conditions.

5 Positive CTL activation can be determined by assaying the cultures for the presence of CTLs that kill radiolabeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed form of the relevant virus or tumor antigen from which the peptide  
10 sequence was derived.

Specificity and MHC restriction of the CTL is determined by testing against different peptide target cells expressing appropriate or inappropriate human MHC class I. The peptides that test positive in the MHC binding assays and  
15 give rise to specific CTL responses are referred to herein as immunogenic peptides.

The immunogenic peptides can be prepared synthetically, or by recombinant DNA technology or from natural sources such as whole viruses or tumors. Although the  
20 peptide will preferably be substantially free of other naturally occurring host cell proteins and fragments thereof, in some embodiments the peptides can be synthetically conjugated to native fragments or particles.

The polypeptides or peptides can be a variety of  
25 lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the  
30 polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be desirable to optimize peptides of the invention to a length of  
35 about 8 to about 10 amino acid residues, commensurate in size with endogenously processed viral peptides or tumor cell peptides that are bound to MHC class I molecules on the cell surface.

Peptides having the desired activity may be modified as necessary to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance, the peptides may be subject to various changes, such as substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. The effect of single amino acid substitutions may also be probed using D-amino acids. Such modifications may be made using well known peptide synthesis procedures, as described in e.g., Merrifield, Science 232:341-347 (1986), Barany and Merrifield, The Peptides, Gross and Meienhofer, eds. (N.Y., Academic Press), pp. 1-284 (1979); and Stewart and Young, Solid Phase Peptide Synthesis, (Rockford, Ill., Pierce), 2d Ed. (1984), incorporated by reference herein.

The peptides can also be modified by extending or decreasing the compound's amino acid sequence, e.g., by the addition or deletion of amino acids. The peptides or analogs of the invention can also be modified by altering the order or composition of certain residues, it being readily appreciated that certain amino acid residues essential for biological activity, e.g., those at critical contact sites or conserved residues, may generally not be altered without an adverse effect on biological activity. The non-critical amino acids need not be limited to those naturally occurring in proteins, such as L- $\alpha$ -amino acids, or their D-isomers, but may include non-natural amino acids as well, such as  $\beta$ - $\gamma$ - $\delta$ -amino acids, as well as many derivatives of L- $\alpha$ -amino acids.

Typically, a series of peptides with single amino acid substitutions are employed to determine the effect of electrostatic charge, hydrophobicity, etc. on binding. For instance, a series of positively charged (e.g., Lys or Arg) or negatively charged (e.g., Glu) amino acid substitutions are made along the length of the peptide revealing different patterns of sensitivity towards various MHC molecules and T cell receptors. In addition, multiple substitutions using small, relatively neutral moieties such as Ala, Gly, Pro, or similar residues may be employed. The substitutions may be homo-oligomers or hetero-oligomers. The number and types of residues which are substituted or added depend on the spacing necessary between essential contact points and certain functional attributes which are sought (e.g., hydrophobicity versus hydrophilicity). Increased binding affinity for an MHC molecule or T cell receptor may also be achieved by such substitutions, compared to the affinity of the parent peptide. In any event, such substitutions should employ amino acid residues or other molecular fragments chosen to avoid, for example, steric and charge interference which might disrupt binding.

Amino acid substitutions are typically of single residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final peptide. Substitutional variants are those in which at least one residue of a peptide has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 2 when it is desired to finely modulate the characteristics of the peptide.

TABLE 2

<u>Original Residue</u>	<u>Exemplary Substitution</u>
Ala	Ser
Arg	Lys, His
Asn	Gln
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Lys; Arg
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; His
Met	Leu; Ile
Phe	Tyr; Trp
Ser	Thr
Thr	Ser
Trp	Tyr; Phe
Tyr	Trp; Phe
Val	Ile; Leu

Substantial changes in function (e.g., affinity for MHC molecules or T cell receptors) are made by selecting substitutions that are less conservative than those in Table 2, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in peptide properties will be those in which (a) hydrophilic residue, e.g. seryl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (c) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

The peptides may also comprise isosteres of two or more residues in the immunogenic peptide. An isostere as defined here is a sequence of two or more residues that can be substituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the  $\alpha$ -carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, Chemistry and Biochemistry of Amino Acids, peptides and Proteins, Vol. VII (Weinstein ed., 1983).

Modifications of peptides with various amino acid mimetics or unnatural amino acids are particularly useful in increasing the stability of the peptide *in vivo*. Stability can be assayed in a number of ways. For instance, peptidases and various biological media, such as human plasma and serum, have been used to test stability. See, e.g., Verhoef et al.,

Eur. J. Drug Metab. Pharmacokin. 11:291-302 (1986). Half  
life of the peptides of the present invention is conveniently  
determined using a 25% human serum (v/v) assay. The protocol  
is generally as follows. Pooled human serum (Type AB,  
5 non-heat inactivated) is delipidated by centrifugation before  
use. The serum is then diluted to 25% with RPMI tissue  
culture media and used to test peptide stability. At  
predetermined time intervals a small amount of reaction  
solution is removed and added to either 6% aqueous  
10 trichloroacetic acid or ethanol. The cloudy reaction sample is  
cooled (4°C) for 15 minutes and then spun to pellet the  
precipitated serum proteins. The presence of the peptides is  
then determined by reversed-phase HPLC using  
stability-specific chromatography conditions.

15 The peptides of the present invention or analogs  
thereof which have CTL stimulating activity may be modified to  
provide desired attributes other than improved serum half  
life. For instance, the ability of the peptides to induce CTL  
activity can be enhanced by linkage to a sequence which  
20 contains at least one epitope that is capable of inducing a T  
helper cell response.

In some embodiments, the T helper peptide is one that  
is recognized by T helper cells in the majority of the  
population. This can be accomplished by selecting amino acid  
25 sequences that bind to many, most, or all of the MHC class II  
molecules. These are known as "loosely MHC-restricted" T  
helper sequences. Examples of amino acid sequences that are  
loosely MHC-restricted include sequences from antigens such as  
Tetanus toxin at positions 830-843 (QYIKANSKFIGITE),  
30 *Plasmodium falciparum* CS protein at positions 378-398  
(DIEKKIAKMEKASSVFNVNS), and *Streptococcus* 18kD protein at  
positions 1-16 (YGAVDSILGGVATYGAA).

Alternatively, it is possible to prepare synthetic  
peptides capable of stimulating T helper lymphocytes, in a  
35 loosely MHC-restricted fashion, using amino acid sequences not  
found in nature. These synthetic compounds called  
Pan-DR-binding epitope (PADRE) are designed on the basis of

their binding activity to most, HLA-DR (human MHC class II) molecules (see, copending application USSN 08/121,101).

Particularly preferred immunogenic peptides/T helper conjugates are linked by a spacer molecule. The spacer is typically comprised of relatively small, neutral molecules, such as amino acids or amino acid mimetics, which are substantially uncharged under physiological conditions. The spacers are typically selected from, e.g., Ala, Gly, or other neutral spacers of nonpolar amino acids or neutral polar amino acids. It will be understood that the optionally present spacer need not comprise the same residues and thus may be a hetero- or homo-oligomer. When present, the spacer will usually be at least one or two residues, more usually three to six residues. Alternatively, the CTL peptide may be linked to the T helper peptide without a spacer.

The immunogenic peptide may be linked to the T helper peptide either directly or via a spacer either at the amino or carboxy terminus of the CTL peptide. The amino terminus of either the immunogenic peptide or the T helper peptide may be acylated. Exemplary T helper peptides include tetanus toxoid 830-843, influenza 307-319, malaria circumsporozoite 382-398 and 378-389.

In some embodiments it may be desirable to include in the pharmaceutical compositions of the invention at least one component which primes CTL. Lipids have been identified as agents capable of priming CTL in vivo against viral antigens. For example, palmitic acid residues can be attached to the alpha and epsilon amino groups of a Lys residue and then linked, e.g., via one or more linking residues such as Gly, Gly-Gly-, Ser, Ser-Ser, or the like, to an immunogenic peptide. The lipidated peptide can then be injected directly in a micellar form, incorporated into a liposome or emulsified in an adjuvant, e.g., incomplete Freund's adjuvant. In a preferred embodiment a particularly effective immunogen comprises palmitic acid attached to alpha and epsilon amino groups of Lys, which is attached via linkage, e.g., Ser-Ser, to the amino terminus of the immunogenic peptide.



As another example of lipid priming of CTL responses, E. coli lipoproteins, such as tripalmitoyl-S-glycerylcysteinylserine ( $P_3$ CSS) can be used to prime virus specific CTL when covalently attached to an appropriate peptide. See, Deres et al., Nature 342:561-564 (1989), incorporated herein by reference. Peptides of the invention can be coupled to  $P_3$ CSS, for example, and the lipopeptide administered to an individual to specifically prime a CTL response to the target antigen. Further, as the induction of neutralizing antibodies can also be primed with  $P_3$ CSS conjugated to a peptide which displays an appropriate epitope, the two compositions can be combined to more effectively elicit both humoral and cell-mediated responses to infection.

In addition, additional amino acids can be added to the termini of a peptide to provide for ease of linking peptides one to another, for coupling to a carrier support, or larger peptide, for modifying the physical or chemical properties of the peptide or oligopeptide, or the like. Amino acids such as tyrosine, cysteine, lysine, glutamic or aspartic acid, or the like, can be introduced at the C- or N-terminus of the peptide or oligopeptide. Modification at the C terminus in some cases may alter binding characteristics of the peptide. In addition, the peptide or oligopeptide sequences can differ from the natural sequence by being modified by terminal- $NH_2$  acylation, e.g., by alkanoyl ( $C_1$ - $C_{20}$ ) or thioglycolyl acetylation, terminal-carboxyl amidation, e.g., ammonia, methylamine, etc. In some instances these modifications may provide sites for linking to a support or other molecule.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984), supra.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982), which is incorporated herein by reference. Thus, fusion proteins which comprise one or more peptide sequences of the invention can be used to present the appropriate T cell epitope.

As the coding sequence for peptides of the length contemplated herein can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981), modification can be made simply by substituting the appropriate base(s) for those encoding the native peptide sequence. The coding sequence can then be provided with appropriate linkers and ligated into expression vectors commonly available in the art, and the vectors used to transform suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are now available. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions and usually a replication system to provide an expression vector for expression in the desired cellular host. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast or mammalian cell hosts may also be used, employing suitable vectors and control sequences.

The peptides of the present invention and pharmaceutical and vaccine compositions thereof are useful for administration to mammals, particularly humans, to treat and/or prevent viral infection and cancer. Examples of

diseases which can be treated using the immunogenic peptides of the invention include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma, lymphoma, CMV and condyloma acuminatum.

5           For pharmaceutical compositions, the immunogenic peptides of the invention are administered to an individual already suffering from cancer or infected with the virus of interest. Those in the incubation phase or the acute phase of infection can be treated with the immunogenic peptides  
10 separately or in conjunction with other treatments, as appropriate. In therapeutic applications, compositions are administered to a patient in an amount sufficient to elicit an effective CTL response to the virus or tumor antigen and to cure or at least partially arrest symptoms and/or  
15 complications. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general  
20 state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization (that is for therapeutic or prophylactic administration) from about 1.0  $\mu$ g to about 5000  $\mu$ g of peptide for a 70 kg patient, followed by boosting dosages of from  
25 about 1.0  $\mu$ g to about 1000  $\mu$ g of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may  
30 generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the relative nontoxic nature of the peptides, it is possible and may be felt desirable by the treating  
35 physician to administer substantial excesses of these peptide compositions.

For therapeutic use, administration should begin at the first sign of viral infection or the detection or surgical

removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting doses until at least symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by  
5 boosting doses may be required.

Treatment of an infected individual with the compositions of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic  
10 infection the compositions are particularly useful in methods for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, for instance, as described herein, the composition can be targeted to them, minimizing need for  
15 administration to a larger population.

The peptide compositions can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers. It is important to provide an amount of immuno-potentiating peptide  
20 in a formulation and mode of administration sufficient to effectively stimulate a cytotoxic T cell response. Thus, for treatment of chronic infection, a representative dose is in the range of about 1.0  $\mu\text{g}$  to about 5000  $\mu\text{g}$ , preferably about 5  $\mu\text{g}$  to 1000  $\mu\text{g}$  for a 70 kg patient per dose. Immunizing doses  
25 followed by boosting doses at established intervals, e.g., from one to four weeks, may be required, possibly for a prolonged period of time to effectively immunize an individual. In the case of chronic infection, administration should continue until at least clinical symptoms or laboratory  
30 tests indicate that the viral infection has been eliminated or substantially abated and for a period thereafter.

The pharmaceutical compositions for therapeutic treatment are intended for parenteral, topical, oral or local administration. Preferably, the pharmaceutical compositions  
35 are administered parenterally, e.g., intravenously, subcutaneously, intradermally, or intramuscularly. Thus, the invention provides compositions for parenteral administration which comprise a solution of the immunogenic peptides

dissolved or suspended in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.8% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be  
5 sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain  
10 pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium  
15 chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of CTL stimulatory peptides of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be  
20 selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a  
25 particular tissue, such as lymphoid tissue, or targeted selectively to infected cells, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In  
30 these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among lymphoid cells, such as monoclonal antibodies which bind to the CD45 antigen, or with other therapeutic or immunogenic  
35 compositions. Thus, liposomes either filled or decorated with a desired peptide of the invention can be directed to the site of lymphoid cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions.

Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

For targeting to the immune cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired immune system cells. A liposome suspension containing a peptide may be administered intravenously, locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the peptide being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of peptides are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an

aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In another aspect the present invention is directed to vaccines which contain as an active ingredient an immunogenically effective amount of an immunogenic peptide as described herein. The peptide(s) may be introduced into a host, including humans, linked to its own carrier or as a homopolymer or heteropolymer of active peptide units. Such a polymer has the advantage of increased immunological reaction and, where different peptides are used to make up the polymer, the additional ability to induce antibodies and/or CTLs that react with different antigenic determinants of the virus or tumor cells. Useful carriers are well known in the art, and include, e.g., thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(lysine:glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are materials well known in the art. And, as mentioned above, CTL responses can be primed by conjugating peptides of the invention to lipids, such as P<sub>3</sub>CSS. Upon immunization with a peptide composition as described herein, via injection, aerosol, oral, transdermal or other route, the immune system of the host responds to the vaccine by producing large amounts of CTLs specific for the desired antigen, and the host becomes at least partially immune to later infection, or resistant to developing chronic infection.

Vaccine compositions containing the peptides of the invention are administered to a patient susceptible to or

otherwise at risk of viral infection or cancer to elicit an immune response against the antigen and thus enhance the patient's own immune response capabilities. Such an amount is defined to be an "immunogenically effective dose." In this use, the precise amounts again depend on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc., but generally range from about 1.0  $\mu\text{g}$  to about 5000  $\mu\text{g}$  per 70 kilogram patient, more commonly from about 10  $\mu\text{g}$  to about 500  $\mu\text{g}$  mg per 70 kg of body weight.

In some instances it may be desirable to combine the peptide vaccines of the invention with vaccines which induce neutralizing antibody responses to the virus of interest, particularly to viral envelope antigens.

For therapeutic or immunization purposes, the peptides of the invention can also be expressed by attenuated viral hosts, such as vaccinia or fowlpox. This approach involves the use of vaccinia virus as a vector to express nucleotide sequences that encode the peptides of the invention. Upon introduction into an acutely or chronically infected host or into a non-infected host, the recombinant vaccinia virus expresses the immunogenic peptide, and thereby elicits a host CTL response. Vaccinia vectors and methods useful in immunization protocols are described in, e.g., U.S. Patent No. 4,722,848, incorporated herein by reference. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover et al. (Nature 351:456-460 (1991)) which is incorporated herein by reference. A wide variety of other vectors useful for therapeutic administration or immunization of the peptides of the invention, e.g., Salmonella typhi vectors and the like, will be apparent to those skilled in the art from the description herein.

Antigenic peptides may be used to elicit CTL ex vivo, as well. The resulting CTL, can be used to treat chronic infections (viral or bacterial) or tumors in patients that do not respond to other conventional forms of therapy, or will not respond to a peptide vaccine approach of therapy. Ex vivo CTL responses to a particular pathogen (infectious agent or tumor antigen) are induced by incubating in tissue culture the



patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate immunogenic peptide. After an appropriate incubation time (typically 1-4 weeks), in which the CTLp are activated and mature and expand  
5 into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell).

The peptides may also find use as diagnostic reagents. For example, a peptide of the invention may be used to  
10 determine the susceptibility of a particular individual to a treatment regimen which employs the peptide or related peptides, and thus may be helpful in modifying an existing treatment protocol or in determining a prognosis for an affected individual. In addition, the peptides may also be  
15 used to predict which individuals will be at substantial risk for developing chronic infection.

The following examples are offered by way of illustration, not by way of limitation.

Example 1Class I antigen isolation

A flow diagram of an HLA-A antigen purification scheme is presented in Figure 1. Briefly, the cells bearing the appropriate allele were grown in large batches (6-8 liters yielding  $\sim 5 \times 10^9$  cells), harvested by centrifugation and washed. All cell lines were maintained in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS) and antibiotics. For large-scale cultures, cells were grown in roller bottle culture in RPMI 1640 with 10% FBS or with 10% horse serum and antibiotics. Cells were harvested by centrifugation at 1500 RPM IEC-CRU5000 centrifuge with 259 rotor and washed three times with phosphate-buffered saline (PBS) (0.01 M  $\text{PO}_4$ , 0.154 M NaCl, pH 7.2).

Cells were pelleted and stored at  $-70^\circ\text{C}$  or treated with detergent lysing solution to prepare detergent lysates. Cell lysates were prepared by the addition of stock detergent solution [1% NP-40 (Sigma) or Renex 30 (Accurate Chem. Sci. Corp., Westbury, NY 11590), 150 mM NaCl, 50 mM Tris, pH 8.0] to the cell pellets (previously counted) at a ratio of 50-100  $\times 10^6$  cells per ml detergent solution. A cocktail of protease inhibitors was added to the premeasured volume of stock detergent solution immediately prior to the addition to the cell pellet. Addition of the protease inhibitor cocktail produced final concentrations of the following: phenylmethylsulfonyl fluoride (PMSF), 2 mM; aprotinin, 5  $\mu\text{g}/\text{ml}$ ; leupeptin, 10  $\mu\text{g}/\text{ml}$ ; pepstatin, 10  $\mu\text{g}/\text{ml}$ ; iodoacetamide, 100  $\mu\text{M}$ ; and EDTA, 3 ng/ml. Cell lysis was allowed to proceed at  $4^\circ\text{C}$  for 1 hour with periodic mixing. Routinely  $5-10 \times 10^9$  cells were lysed in 50-100 ml of detergent solution. The lysate was clarified by centrifugation at  $15,000 \times g$  for 30 minutes at  $4^\circ\text{C}$  and subsequent passage of the supernatant fraction through a  $0.2 \mu$  filter unit (Nalgene).

The HLA-A antigen purification was achieved using affinity columns prepared with mAb-conjugated Sepharose beads. For antibody production, cells were grown in RPMI with 10% FBS in large tissue culture flasks (Corning 25160-225).

Antibodies were purified from clarified tissue culture medium by ammonium sulfate fractionation followed by affinity chromatography on protein-A-Sepharose (Sigma). Briefly, saturated ammonium sulfate was added slowly with stirring to the tissue culture supernatant to 45% (volume to volume) overnight at 4°C to precipitate the immunoglobulins. The precipitated proteins were harvested by centrifugation at 10,000 x g for 30 minutes. The precipitate was then dissolved in a minimum volume of PBS and transferred to dialysis tubing (Spectro/Por 2, Mol. wt. cutoff 12,000-14,000, Spectrum Medical Ind.). Dialysis was against PBS ( $\geq 20$  times the protein solution volume) with 4-6 changes of dialysis buffer over a 24-48 hour period at 4°C. The dialyzed protein solution was clarified by centrifugation (10,000 x g for 30 minutes) and the pH of the solution adjusted to pH 8.0 with 1N NaOH. Protein-A-Sepharose (Sigma) was hydrated according to the manufacturer's instructions, and a protein-A-Sepharose column was prepared. A column of 10 ml bed volume typically binds 50-100 mg of mouse IgG.

The protein sample was loaded onto the protein-A-Sepharose column using a peristaltic pump for large loading volumes or by gravity for smaller volumes (<100 ml). The column was washed with several volumes of PBS, and the eluate was monitored at A280 in a spectrophotometer until base line was reached. The bound antibody was eluted using 0.1 M citric acid at suitable pH (adjusted to the appropriate pH with 1N NaOH). For mouse IgG-1 pH 6.5 was used for IgG2a pH 4.5 was used and for IgG2b and IgG3 pH 3.0 was used. 2 M Tris base was used to neutralize the eluate. Fractions containing the antibody (monitored by A280) were pooled, dialyzed against PBS and further concentrated using an Amicon Stirred Cell system (Amicon Model 8050 with YM30 membrane). The anti-A2 mAb, BB7.2, was useful for affinity purification.

The HLA-A antigen was purified using affinity columns prepared with mAb-conjugated Sepharose beads. The affinity columns were prepared by incubating protein-A-Sepharose beads (Sigma) with affinity-purified mAb as described above. Five to 10 mg of mAb per ml of bead is the preferred ratio. The

mAb bound beads were washed with borate buffer (borate buffer: 100 mM sodium tetraborate, 154 mM NaCl, pH 8.2) until the washes show A280 at based line. Dimethyl pimelimidate (20 mM) in 200 mM triethanolamine was added to covalently crosslink the bound mAb to the protein-A-Sepharose (Schneider et al., J. Biol. Chem. 257:10766 (1982)). After incubation for 45 minutes at room temperature on a rotator, the excess crosslinking reagent was removed by washing the beads twice with 10-20 ml of 20 mM ethanolamine, pH 8.2. Between each one the slurry was placed on a rotator for 5 minutes at room temperature. The beads were washed with borate buffer and with PBS plus 0.02% sodium azide.

The cell lysate ( $5-10 \times 10^9$  cell equivalents) was then slowly passed over a 5-10 ml affinity column (flow rate of 0.1-0.25 ml per minute) to allow the binding of the antigen to the immobilized antibody. After the lysate was allowed to pass through the column, the column was washed sequentially with 20 column volumes of detergent stock solution plus 0.1% sodium dodecyl sulfate, 20 column volumes of 0.5 M NaCl, 20 mM Tris, pH 8.0, and 10 column volumes of 20 mM Tris, pH 8.0. The HLA-A antigen bound to the mAb was eluted with a basic buffer solution (50 mM diethylamine in water). As an alternative, acid solutions such as 0.15-0.25 M acetic acid were also used to elute the bound antigen. An aliquot of the eluate (1/50) was removed for protein quantification using either a colorimetric assay (BCA assay, Pierce) or by SDS-PAGE, or both. SDS-PAGE analysis was performed as described by Laemmli (Laemmli, U.K., Nature 227:680 (1970)) using known amounts of bovine serum albumin (Sigma) as a protein standard. Allele specific antibodies were used to purify the specific MHC molecule. In the case of HLA-A2, the mAb BB7.2 was used.

#### Example 2

##### Isolation and sequencing of naturally processed peptides

For the HLA-A preparations derived from the base (50 mM diethylamine) elution protocol, the eluate was immediately neutralized with 1 N acetic acid to pH 7.0-7.5. The neutralized eluate was concentrated to a volume of 1-2 ml in

an Amicon stirred cell [Model 8050, with YM3 membranes (Amicon)]. Ten ml of ammonium acetate (0.01 M, pH 8.0) was added to the concentrator to remove the non-volatile salts, and the sample was concentrated to approximately 1 ml. A  
5 small sample (1/50) was removed for protein quantitation as described above. The remainder was recovered into a 15 ml polypropylene conical centrifuge tube (Falcon, 2097) (Becton Dickinson). Glacial acetic acid was added to obtain a final concentration of 10% acetic acid. The acidified sample was  
10 placed in a boiling water bath for 5 minutes to allow for the dissociation of the bound peptides. The sample was cooled on ice, returned to the concentrator and the filtrate was collected. Additional aliquots of 10% acetic acid (1-2 ml) were added to the concentrator, and this filtrate was pooled  
15 with the original filtrate. Finally, 1-2 ml of distilled water was added to the concentrator, and this filtrate was pooled as well.

The retentate contains the bulk of the HLA-A heavy chain and  $\beta_2$ -microglobulin, while the filtrate contains the  
20 naturally processed bound peptides and other components with molecular weights less than about 3000. The pooled filtrate material was lyophilized in order to concentrate the peptide fraction. The sample was then ready for further analysis.

For HPLC (high performance liquid chromatography)  
25 separation of the peptide fractions, the lyophilized sample was dissolved in 50  $\mu$ l of distilled water, or into 0.1% trifluoroacetic acid (TFA) (Applied Biosystems) in water and injected to a C18 reverse-phase narrow bore column (Beckman C18 Ultrasphere, 10 x 250 mm), using a gradient system  
30 described by Stone and Williams (Stone, K.L. and Williams K.R., in, Macromolecular Sequencing and Synthesis; Selected Methods and Applications, A.R. Liss, New York, 1988, pp. 7-24. Buffer A was 0.06% TFA in water (Burdick-Jackson) and buffer B was 0.052% TFA in 80% acetonitrile (Burdick-Jackson). The  
35 flow rate was 0.250 ml/minute with the following gradient: 0-60 min., 2-37.5% B; 60-95 min., 37.5-75% B; 95-105 min., 75-98% B. The Gilson narrow bore HPLC configuration is

particularly useful for this purpose, although other configurations work equally well.

5 A large number of peaks were detected by absorbance at 214 nm, many of which appear to be of low abundance. Whether a given peak represents a single peptide or a peptide mixture was not determined. Pooled fractions were then sequenced to determine motifs specific for each allele as described below.

10 Pooled peptide fractions, prepared as described above were analyzed by automated Edman sequencing using the Applied Biosystems Model 477A automated sequencer. The sequencing method is based on the technique developed by Pehr Edman in the 1950s for the sequential degradation of proteins and peptides to determine the sequence of the constituent amino acids.

15 The protein or peptide to be sequenced was held by a 12-mm diameter porous glass fiber filter disk in a heated, argon-purged reaction chamber. The filter was generally pre-treated with BioBrene Plus<sup>TM</sup> and then cycled through one or more repetitions of the Edman reaction to reduce contaminants and improve the efficiency of subsequent sample sequencing. 20 Following the pre-treatment of the filter, a solution of the sample protein or peptide (10 pmol-5 nmol range) was loaded onto the glass filter and dried. Thus, the sample was left embedded in the film of the pre-treated disk. Covalent 25 attachment of the sample to the filter was usually not necessary because the Edman chemistry utilized relatively apolar solvents, in which proteins and peptides are poorly soluble.

30 Briefly, the Edman degradation reaction has three steps: coupling, cleavage, and conversion. In coupling step, phenylisothiocyanate (PITC) is added. The PITC reacts quantitatively with the free amino-terminal amino acid of the protein to form the phenylthiocarbamyl-protein in a basic environment. After a period of time for the coupling step, 35 the excess chemicals are extracted and the highly volatile organic acid, trifluoroacetic acid, TFA, is used to cleave the PITC-coupled amino acid residue from the amino terminus of the protein yielding the anilinothiazolinone (ATZ) derivative of

the amino acid. The remaining protein/peptide is left with a new amino terminus and is ready for the next Edman cycle. The ATZ amino acid is extracted and transferred to a conversion flask, where upon addition of 25% TFA in water, the ATZ amino acid is converted to the more stable phenylthiohydantoin (PTH) amino acid that can be identified and quantified following automatic injection into the Model 120 PTH Analyzer which uses a microbore C-18 reverse-phase HPLC column for the analysis.

In the present procedures, peptide mixtures were loaded onto the glass filters. Thus, a single amino acid sequence usually does not result. Rather, mixtures of amino acids in different yield are found. When the particular residue is conserved among the peptides being sequenced, increased yield for that amino acid is observed.

### Example 3

#### Definition of an A2.1 specific motif

In one case, pooled peptide fractions prepared as described in Example 2 above were obtained from HLA-A2.1 homozygous cell lines, for example, JY. The pooled fractions were HPLC fractions corresponding to 7% to 45% CH<sub>3</sub>CN. For this class I molecule, this region of the chromatogram was most abundant in peptides. Data from independent experiments were averaged as described below.

The amino acid sequence analyses from four independent experiments were analyzed and the results are shown in Table 3. For each position except the first, the data were analyzed by modifying the method described by Falk et al., supra, to allow for comparison of experiments from different HLA types. This modified procedure yielded quantitative yet standardized values while allowing the averaging of data from different experiments involving the same HLA type.

The raw sequenator data was converted to a simple matrix of 10 rows (each representing one Edman degradation cycle) and 16 columns (each representing one of the twenty amino acids; W, C, R and H were eliminated for technical reasons. The data corresponding to the first row (first cycle) was not considered further because, this cycle is

usually heavily contaminated by free amino acids.). The values of each row were summed to yield a total pmoles value for that particular cycle. For each row, values for each amino acid were then divided by the corresponding total yield value, to determine what fraction of the total signal is attributable to each amino acid at each cycle. By doing so, an "Absolute Frequency" table was generated. This absolute frequency table allows correction for the declining yields of each cycle.



TABLE 3  
A2.1: POOL SEQUENCING FREQUENCY

	pos. 1	pos. 2	pos. 3	pos. 4	pos. 5	pos. 6	pos. 7	pos. 8	pos. 9	pos. 10
A	-	0.65	1.25	0.85	0.95	0.77	1.21	1.16	1.15	1.25
G	-	0.84	0.96	1.29	1.22	0.89	0.78	1.05	0.98	1.48
D	-	0.84	1.11	1.70	1.03	0.83	0.82	0.84	0.82	1.19
E	-	0.38	0.59	1.73	1.10	0.82	1.05	1.45	0.87	0.88
R	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-
K	-	0.63	0.65	0.89	1.66	1.09	0.89	1.35	0.82	0.87
L	-	2.65*	1.11	0.45	0.57	1.00	0.69	0.59	0.92	0.77
V	-	0.78	0.69	0.60	0.79	1.38	1.24	0.84	1.69	1.27
I	-	1.06	1.20	0.53	0.93	1.49	1.15	0.76	0.88	0.54
M	-	1.93	1.91	0.62	0.71	0.68	0.88	0.54	0.73	0.22
Y	-	0.28	1.41	0.65	1.32	0.78	1.34	1.21	1.00	0.79
F	-	0.76	1.46	0.69	1.16	1.00	1.07	1.09	0.78	0.73
W	-	-	-	-	-	-	-	-	-	-
Q	-	0.60	0.84	0.92	0.95	0.90	1.16	1.63	1.00	1.00
N	-	0.39	0.76	1.17	1.28	1.08	1.07	1.28	0.96	0.42
S	-	1.13	1.50	1.33	0.87	0.77	0.71	0.92	0.77	0.58
T	-	0.62	0.90	0.94	0.95	1.21	1.07	1.60	0.71	0.57
C	-	-	-	-	-	-	-	-	-	-
P	-	0.54	0.78	1.44	1.15	1.09	1.30	0.87	0.81	1.01

\* 

Starting from the absolute frequency table, a "relative frequency" table was then generated to allow comparisons among different amino acids. To do so the data from each column was summed, and then averaged. Then, each value was divided next by the average column value to obtain relative frequency values. These values quantitate, in a standardized manner, increases and decreases per cycle, for each of the different sixteen amino acid types. Tables generated from data from different experiments can thus be added together to generate average relative frequency values (and their standard deviations). All standard deviations can then be averaged, to estimate a standard deviation value applicable to the samples from each table. Any particular value exceeding 1.00 by more than two standard deviations is considered to correspond to a significant increase.

#### Example 4

##### Quantitative Binding Assays

Using isolated MHC molecules prepared as described in Example 2, above, quantitative binding assays were performed. Briefly, indicated amounts of MHC as isolated above were incubated in 0.05% NP40-PBS with ~5 nM of radiolabeled peptides in the presence of 1-3  $\mu\text{M}$   $\beta_2\text{M}$  and a cocktail of protease inhibitors (final concentrations 1 mM PMSF, 1.3 mM 1.10 Phenanthroline, 73  $\mu\text{M}$  Pepstatin A, 8 mM EDTA, 200  $\mu\text{M}$  N- $\alpha$ -p-tosyl-L-Lysine Chloromethyl ketone). After various times, free and bound peptides were separated by TSK 2000 gel filtration, as described previously in A. Sette et al., J. Immunol. 148:844 (1992), which is incorporated herein by reference. Peptides were labeled by the use of the Chloramine T method Buus et al., Science 235:1352 (1987), which is incorporated herein by reference.

The HbC 18-27 peptide HLA binding peptide was radiolabeled and offered (5-10 nM) to 1  $\mu\text{M}$  purified HLA A2.1. After two days at 23°C in presence of a cocktail of protease inhibitors and 1-3  $\mu\text{M}$  purified human  $\beta_2\text{M}$ , the percent of MHC class I bound radioactivity was measured by size exclusion chromatography, as previously described for class II peptide

binding assays in Sette et al., in Seminars in Immunology, Vol. 3, Geftter, ed. (W.B. Saunders, Philadelphia, 1991), pp 195-202, which is incorporated herein by reference. Using this protocol, high binding (95%) was detected in all cases in the presence of purified HLA A2.1 molecules.

To explore the specificity of binding, we determined whether the binding was inhibitable by excess unlabeled peptide, and if so, what the 50% inhibitory concentration (IC50%) might be. The rationale for this experiment was threefold. First, such an experiment is crucial in order to demonstrate specificity. Second, a sensitive inhibition assay is the most viable alternative for a high throughput quantitative binding assay. Third, inhibition data subjected to Scatchard analysis can give quantitative estimates of the equilibrium constant (K) of interaction and the fraction of receptor molecules capable of binding ligand (% occupancy). For instance, in analysis of an inhibition curve for the interaction of the peptide HBC 18-27 with A2.1, the IC50% was determined to be 25 nM. Further experiments were conducted to obtain Scatchard plots. For HBC 18-27/A2.1, six different experiments using six independent MHC preparations yielded a  $K_D$  of  $15.5 \pm 9.9 \times 10^{-9}$  M and occupancy values of 6.2% ( $\pm 1.4$ ).

Several reports have demonstrated that class I molecules, unlike class II, are highly selective with regard to the size of the peptide epitope that they recognize. The optimal size varies between 8 and 10 residues for different peptides and different class I molecules, although MHC binding peptides as long as 13 residues have been identified. To verify the stringent size requirement, a series of N- and C-terminal truncation/extension analogs of the peptide HBC 18-27 were synthesized and tested for A2.1 binding. Previous studies had demonstrated that the optimal size for CTL recognition of this peptide was the 10-mer HBC18-27 (Sette et al. supra). It was found that removal or addition of a residue at the C terminus of the molecule resulted in a 30 to 100-fold decrease in binding capacity. Further removal or addition of another residue completely obliterated binding. Similarly, at the N-terminus of the molecule, removal or

deletion of one residue from the optimal HBc 18-27 peptide completely abrogated A2.1 binding.

Throughout this disclosure, results have been expressed in terms of IC50's. Given the conditions in which our assays are run (i.e., limiting MHC and labeled peptide concentrations), these values approximate  $K_D$  values. It should be noted that IC50 values can change, often dramatically, if the assay conditions are varied, and depending on the particular reagents used (e.g., Class I preparation, etc.). For example, excessive concentrations of MHC will increase the apparent measured IC50 of a given ligand.

An alternative way of expressing the binding data, to avoid these uncertainties, is as a relative value to a reference peptide. The reference peptide is included in every assay. As a particular assay becomes more, or less, sensitive, the IC50's of the peptides tested may change somewhat. However, the binding relative to the reference peptide will not change. For example, in an assay run under conditions such that the IC50 of the reference peptide increases 10-fold, all IC50 values will also shift approximately ten-fold. Therefore, to avoid ambiguities, the assessment of whether a peptide is a good, intermediate, weak, or negative binder should be based on its IC50, relative to the IC50 of the standard peptide.

The reference peptide for the HLA-A2.1 assays described herein is referred to as 941.01 having a sequence of FLPSDYFPSV. An average IC50 of 5 (nM) was observed under the assay conditions utilized.

If the IC50 of the standard peptide measured in a particular assay is different from that reported in the table, then it should be understood that the threshold values used to determine good, intermediate, weak, and negative binders should be modified by a corresponding factor. For example, if in an A2.1 binding assay, the IC50 of the A2.1 standard (941.01) were to be measured as 8 nM instead of 5 nM, then a peptide ligand would be called a good binder only if it had an

IC50 of less than 80 nM (i.e., 8nM x 0.1), instead of the usual cut-off value of 50 nM.

#### Example 5

##### 5                   HLA-A2.1 Binding Motif and Algorithm

          The structural requirements for peptide binding to A2.1 have been defined for both, 9-mer and 10-mer peptides. Two approaches have been used. The first approach referred to as the "poly-A approach" uses a panel of single amino acid  
10       substitutions of a 9-mer prototype poly-A binder (ALAKAAAV) that is tested for A2.1 binding using the methods of Example 4 above to examine the degree of degeneracy of the anchor-positions and the possible influence of non-anchor positions on A2.1 binding.

15               The second approach, the "Motif-Library approach", uses a large library of peptides selected from sequences of potential target molecules of viral and tumor origin and tested for A2.1 binding using the methods in Example 4 above. The frequencies by which different amino-acids occurred at each  
20       position in good binders and non-binders were analysed to further define the role of non-anchor positions in 9-mers and 10-mers.

#### A2.1 binding of peptide 9-mers

25               Poly A Approach A poly-A 9-mer peptide, containing the A2.1 motif L (Leu) in position 2 and V (Val) in position 9 was chosen as a prototype binder. A K (Lys) residue was included in position 4 to increase solubility. A panel of 91 single amino-acid substitution analogues of the prototype  
30       parental 9-mer was synthesized and tested for A2.1 binding (Table 4). Shaded areas mark analogs with a greater than 10-fold reduction in binding capacity relative to the parental peptide. A reduction in binding greater than 100-fold is indicated by hyphenation.

35               Anchor-Positions 2 and 9 in poly-A Analogs The effect of single-amino-acid substitutions at the anchor positions 2 and 9 was examined first. Most substitutions in these positions had profound detrimental effects on binding

capacity, thus confirming their role for binding. More specifically, in position 2 only L and M bound within a 10-fold range ("preferred residues"). Residues with similar characteristics, such as I, V, A, and T were tolerated, but  
5 bound 10 to 100-fold less strongly than the parental peptide. All the remaining substitutions (residues S, N, D, F, C, K, G, and P) were not tolerated and decreased binding by more than 100-fold. Comparably stringent requirements were observed for  
10 position 9, where V, L and I were preferred and A and M are tolerated, while the residues T, C, N, F, and Y virtually abolished binding. According to this set of peptides, an optimal 2-9 motif could be defined with L, M in position 2 and V, I, or L in position 9.

TABLE 4  
A2.1: BINDING OF ANALOGS OF A MOTIF-CONTAINING POLY A PEPTIDE

	pos. 1 A	pos. 2 L	pos. 3 A	pos. 4 K	pos. 5 A	pos. 6 A	pos. 7 A	pos. 8 A	pos. 9 V
A	1.00	0.013	1.00		1.00	1.00	1.00	1.00	0.070
G	0.46	-*			0.63	0.12		0.57	
D	-	-	0.93	0.74	0.51	0.10			
E	0.012		0.68	1.53	0.62	0.15	0.28	0.26	
R						0.080			
H								0.24	
K	0.54		0.062	1.00	0.39		0.50	0.11	0.11
L		1.00	0.46		0.99		0.76	0.90	1.00
V	0.47	0.051	0.15	1.12		0.44	0.49	0.30	0.18
I	0.41	0.063				1.12			
M		0.43	0.66						0.024
Y	0.75		0.62		0.94	0.41	1.40	0.43	-
F	1.10		0.95			1.76		0.49	-
W									
Q			0.34		0.32		0.19	0.41	
N			0.37	0.97	1.24		0.97	0.31	
S	0.44								
T	0.26	0.011		0.98			0.28	0.37	
C				1.53		0.84			
P			0.25	1.07		0.84	0.63	0.55	

Ratio  $\leq 0.1$   
Ratio  $\leq 0.01$

\*

Non-Anchor Positions 1 and 3-8 in poly-A Analogs All non-anchor positions were more permissive to different substitutions than the anchor-positions 2 and 9, i.e most residues were tolerated. Significant decreases in binding were observed for some substitutions in distinct positions. More specifically, in position 1 a negative charge (residues D and E) or a P greatly reduced the binding capacity. Most substitutions were tolerated in position 3 with the exception of the residue K. Significant decreases were also seen in position 6 upon introduction of either a negative charge (D, E) or a positively charged residue (R). A summary of these effects by different single amino acid substitutions is given in Table 5.



TABLE 5

Summary

A2.1

Poly-A

5	AA position	(+)	(+/-)	(-)
	1	FAYKVGSIT		EDP
	2	LM	VITA	SNDFCKGP
	3	AFDEMYLSNPV	K	
	4	CEVPATSD		
10	5	NALYGEDKQ		
	6	FIAPCVYEG	DR	
	7	YANLPVETQ		
	8	ALGPFYQTNVEHK		
15	9	VIL	AM	TCNFY
		Ratio > 0.1	Ratio 0.01-0.1	Ratio < 0.01

The Motif-Library Approach To further evaluate the importance of non-anchor positions for binding, peptides of potential target molecules of viral and tumor origin were scanned for the presence of sequences containing optimal 2-9 anchor motifs. A set of 161 peptides containing a L or M in position 2 and a V, L or I in position 9 was selected, synthesized and tested for binding (see Example 6). Only 11.8% of these peptides bind with high affinity (ratio  $\geq 0.10$ ; 22.4% were intermediate binders (ratio  $\geq 0.1$ ). As many as 36% were weak binders (ratio  $< 0.01 - 0.0001$ ), and 31% were non-binders (ratio  $< 0.0001$ ). The high number of non-binders containing optimal anchor-motifs indicates that in this set of peptides positions other than the 2-9 anchors influence A2.1 binding capacity. Appendix 1 sets forth all of the peptides having the 2-9 motif used for this analysis and the binding data for those peptides.

To define the influence on non-anchor positions more specifically, the frequency of occurrence of each amino acid

in each of the non-anchor positions was calculated for the good and intermediate binders on one hand and non-binders on the other hand. Amino acids of similar chemical characteristic were grouped together. Weak binders were not considered for the following analysis. The frequency of occurrence of each amino acid in each of the non-anchor positions was calculated for the good binders and non-binders (Table 6).

Several striking trends become apparent. For example in position 1, only 3.6% of the A2.1 binders and as much as 35% of the non-binders carried a negative charge (residues D and E). This observation correlates well with previous findings in the set of poly-A analogs, where a D or E substitution greatly affected binding. Similarly, the residue P was 8 times more frequent in non-binders than in good binders. Conversely, the frequencies of aromatic residues (Y, F, W) were greatly increased in A2.1 binders as compared to non-binders.

TABLE 6

A.2.1 9-mer PEPTIDES  
 NUMBER OF PEPTIDES  
 GOOD BINDERS 161 11.8%  
 INTERMEDIATE BINDERS 36 22.4%  
 WEAK BINDERS 58 36.0%  
 NON-BINDERS 48 29.8%

	1+	1-2+	2-3+	3-4+	4-5+	5-6+	6-7+	7-8+	8-9+	9-
A	5.5	2.1	0.0	4.2	8.3	8.3	6.3	2.1	12.5	0.0
G	7.3	2.1	0.0	8.3	8.3	8.3	8.3	12.5	8.3	0.0
D,E	3.6	35.4	0.0	12.5	16.7	12.5	8.3	16.7	10.4	0.0
R,H,K	12.7	4.2	0.0	16.7	16.7	10.4	20.8	10.4	12.5	0.0
L,V,I,M	38.2	12.5	100.0	18.8	16.7	29.2	22.9	25.0	18.8	100.0
Y,F,W	14.5	2.1	0.0	4.2	8.3	2.1	8.3	8.3	8.3	0.0
Q,N	7.3	14.8	0.0	14.6	10.4	10.4	10.4	8.3	16.7	0.0
S,T,C	9.1	12.5	0.0	10.4	4.2	16.7	12.5	12.5	18.8	0.0
P	1.8	14.6	0.0	10.4	12.5	2.1	2.1	6.3	0.0	0.0
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Following this approach, amino acids of similar structural characteristics were grouped together. Then, the frequency of each amino acid group in each position was calculated for binders versus non-binders (Table 7). Finally, the frequency in the binders group was divided by the frequency in the non-binders to obtain a "frequency ratio". This ratio indicates whether a given amino-acid or group of residues occurs in a given position preferentially in good binders (ratio >1) or in non-binders (ratio <1).

TABLE 7  
A2.1 9-mer PEPTIDES

NUMBER OF PEPTIDES	161
GOOD BINDERS	19 11.8%
INTERMEDIATE BINDERS	36 22.4%
WEAK BINDERS	58 36.0%
NON-BINDERS	48 29.8%

	pos. 1 ratio	pos. 2 ratio	pos. 3 ratio	pos. 4 ratio	pos. 5 ratio	pos. 6 ratio	pos. 7 ratio	pos. 8 ratio	pos. 9 ratio
A	2.6	NA	0.9	0.9	0.7	0.9	4.4	0.3	NA
G	3.5	NA	0.4	1.1	1.1	1.3	0.4	0.4	NA
D,E	0.1	NA	0.0	0.7	0.3	0.7	0.1	0.9	NA
R,H,K	3.1	NA	0.2	1.0	0.9	0.1	0.0	1.3	NA
L,V,I,M	3.1	1.0	1.8	0.5	0.9	1.3	1.2	1.7	1.0
Y,F,W	7.0	NA	5.2	0.9	8.7	2.0	2.3	2.6	NA
Q,N	0.5	NA	0.4	1.2	0.9	1.0	0.7	0.3	NA
S,T,C	0.7	NA	1.9	4.8	0.9	1.2	1.2	1.1	NA
P	0.1	NA	0.7	0.7	2.6	1.7	2.9	+++	NA

+++ indicates that there were no negative binders

Different Residues Influence A2.1 Binding In order to analyse the most striking influences of certain residues on A2.1 binding, a threshold level was set for the ratios described in Table 7. Residues showing a more than 4-fold greater frequency in good binders were regarded as preferred residues (+). Residues showing a 4-fold lower frequency in A2.1 binders than in non-binders were regarded as disfavored residues (-). Following this approach, residues showing the most prominent positive or negative effects on binding are listed in Table 8.

This table identifies the amino acid groups which influence binding most significantly in each of the non-anchor positions. In general, the most negative effects were observed with charged amino acids. In position 1, negatively, charged amino acids were not observed in good binders, i.e., those amino acids were negative binding residues at position 1. The opposite was true for position 6 where only basic amino acids were detrimental for binding i.e., were negative binding residues. Moreover, both acidic and basic amino acids were not observed in A2.1 binders in positions 3 and 7. A greater than 4-fold increased frequency of non-binders was found when P was in position 1.

TABLE 8

Summary of A2.1 Motif-Library, 9-mers

AA POSITION	(+)	(-)
1	(YFW)	P, (DE)
2	Anchor	
3	(YFW)	(DE), (RKH)
4	(STC)	
5	(YFW)	
6		(RKH)
7	A	(RKH), (DE)
8		
9	Anchor	

(+) = Ratio  $\geq$  4-fold (-) = Ratio  $\leq$  0.25

Aromatic residues were in general favored in several of the non-anchor positions, particularly in positions 1, 3, and 5. Small residues like S, T, and C were favored in position 4 and A was favored in position 7.

An Improved A2.1 9-mer Motif The data described above was used to derive a stringent A2.1 motif. This motif is based in significant part on the effects of the non-anchor positions 1 and 3-8. The uneven distribution of amino acids at different positions is reflective of specific dominant negative binding effects of certain residues, mainly charged

ones, on binding affinity. A series of rules were derived to identify appropriate anchor residues in positions 2 and 9 and negative binding residues at positions 1 and 3-8 to enable selection of a high affinity binding immunogenic peptide.

5 These rules are summarized in Table 9.

To validate the motif defined above and shown in Table 9 published sequences of peptides that have been naturally processed and presented by A2.1 molecules were analysed (Table 10). Only 9-mer peptides containing the 2-9 anchor residues  
10 were considered.

When the frequencies of these peptides were analysed, it was found that in general they followed the rules summarized in Table 9. More specifically, neither acidic amino acids nor P were found in position 1. Only one acidic amino acid and no  
15 basic amino acids were found in position 3. Positions 6 and 7 showed no charged residues. Acidic amino acids, however, were frequently found in position 8, where they are tolerated, according to our definition of the A2.1 motif. The analysis of the sequences of naturally processed peptides therefore  
20 reveals that >90% of the peptides followed the defined rules for a complete motif.

Thus the data confirms a role of positions other than the anchor positions 2 and 9 for A2.1 binding. Most of the deleterious effects on binding are induced by charged amino  
25 acids in non-anchor positions, i.e. negative binding residues occupying positions 1, 3, 6 or 7.

TABLE 9  
A2.1 MOTIF FOR 9-MER PEPTIDES

5	AA Position	(+)	(-)
	1		acidic amino-acids and P
	2	Anchor: L, M, (I,V,A,T)	
	3		acidic and basic amino-acids
	4		
10	5		
	6		basic amino-acids
	7		acidic and basic amino-acids
	8		
15	9	Anchor: V, I, L (A,M)	

TABLE 10

## A2.1 naturally processed peptides

1	2	3	4	5	6	7	8	9	A2.1 binding
A	L	X	G	G	X	V	N	V	ND
L	L	D	V	P	T	A	A	V	ND
G	X	V	P	F	X	V	S	V	0.41
S	L	L	P	A	I	V	E	L	0.19
S	X	X	V	R	A	X	E	V	ND
Y	M	N	G	T	M	S	Q	V	ND
K	X	N	E	P	V	X	X	X	ND
Y	L	L	P	A	I	V	H	I	0.26
A	X	W	G	F	F	P	V	X	ND
T	L	W	V	D	P	Y	E	V	0.23
G	X	V	P	F	X	V	S	V	0.41

A2.1 Binding of Peptide 10-mers

The "Motif-Library" Approach Previous data clearly indicated that 10-mers can also bind to HLA molecules even if with a somewhat lower affinity than 9-mers. For this reason we expanded our analysis to 10-mer peptides.

Therefore, a "Motif-Library" set of 170 peptide 10-mers containing optimal motif-combinations was selected from known target molecule sequences of viral and tumor origin and analysed as described above for 9-mers. In this set we found 5.9% good binders, 17.1% intermediate binders, 41.2% weak binders and 35.9% non-binders. The actual sequences, origin and binding capacities of this set of peptides are included as Appendix 2. This set of 10-mers was used to determine a) the rules for 10-mer peptide binding to A2.1, b) the similarities or differences to rules defined for 9-mers, and c) if an insertion point can be identified that would allow for a superimposable common motif for 9-mers and 10-mers.



Amino-acid frequencies and frequency ratios for the various amino-acid groups for each position were generated for 10-mer peptides as described above for 9-mer peptides and are also shown in Tables 11 and 12, respectively for grouped  
5 residues.

A summary of preferred versus disfavored residues and of the rules derived for the 10-mers in a manner analogous to that used for 9-mers, is also listed in Tables 13 and 14, respectively.

10 When the frequency-ratios of different amino-acid groups in binders and non-binders at different positions were analysed and compared to the corresponding ratios for the 9-mers, both striking similarities and significant differences emerged (Table 15). At the N-terminus and the C-termini of 9-  
15 mers and 10-mers, similarities predominate. In position 1 for example, in 10-mers again the P residue and acidic amino acids were not tolerated. In addition at position 1 in 10-mers aromatic residues were frequently observed in A2.1 binders. In position 3, acidic amino acids were frequently associated with  
20 poor binding capacity in both 9-mers and 10-mers. Interestingly, however, while in position 3 aromatic residues

TABLE 11

A2.1 10-mer Peptides		NUMBER OF PEPTIDES		WEAK BINDERS		70 41.2%		NON-BINDERS		61 35.9%	
GOOD BINDERS		10	5.9%	INTERMEDIATE BINDERS		29	17.1%				
		1+	1-	2+	2-	3+	3-	4+	4-	5+	5-
A		2.6	0.0	0.0	0.0	10.3	3.3	2.6	11.5	5.1	3.3
G		7.7	9.8	0.0	0.0	7.7	16.4	15.4	3.3	5.1	6.6
D,E		0.0	23.0	0.0	0.0	2.6	16.4	7.7	13.1	2.6	9.8
R,H,K		7.7	6.6	0.0	0.0	5.1	16.4	2.8	16.0	10.3	14.8
L,V,I,M		48.7	16.4	100.0	100.0	33.3	3.3	23.1	23.0	30.8	24.6
Y,F,W		12.8	0.0	0.0	0.0	12.6	4.9	15.4	4.9	17.9	4.9
Q,N		10.3	9.6	0.0	0.0	7.7	8.2	7.7	9.8	7.7	9.8
S,T,C		10.3	11.5	0.0	0.0	15.4	18.0	12.6	11.5	20.5	19.7
P		0.0	23.0	0.0	0.0	5.1	13.1	12.6	4.9	0.0	6.6
		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

		6+	6-	7+	7-	8+	8-	9+	9-	10+	10-
A		7.7	13.1	10.3	8.2	7.7	4.9	2.6	4.9	0.0	0.0
G		10.3	1.6	17.9	6.6	7.7	11.5	7.7	9.8	0.0	0.0
D,E		10.3	9.6	5.1	15.4	0.0	16.4	5.1	13.1	0.0	0.0
R,H,K		7.7	19.7	2.6	14.8	0.0	29.5	2.6	16.4	0.0	0.0
L,V,I,M		30.8	14.5	25.5	18.0	23.1	4.9	12.8	16.4	100.0	100.0
Y,F,W		7.7	13.1	12.8	6.2	23.1	1.6	20.5	9.6	0.0	0.0
Q,N		2.6	3.3	5.1	8.2	2.6	6.6	7.7	11.5	0.0	0.0
S,T,C		17.9	19.7	17.9	13.1	20.5	16.4	33.3	11.5	0.0	0.0
P		5.1	4.9	2.6	5.6	15.4	8.2	7.7	6.6	0.0	0.0
		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

TABLE 12

## A2.1 10-mer Peptides

NUMBER OF PEPTIDES  
 GOOD BINDERS 170 5.9%  
 INTERMEDIATE BINDERS 10 17.1%  
 WEAK BINDERS 29 41.2%  
 NON-BINDERS 70 35.9%  
 61

	pos. 1 ratio	pos. 2 ratio	pos. 3 ratio	pos. 4 ratio	pos. 5 ratio	pos. 6 ratio	pos. 7 ratio	pos. 8 ratio	pos. 9 ratio	pos. 10 ratio
A	+++	NA	3.1	0.2	1.8	0.6	1.3	1.6	0.5	NA
G	0.8	NA	0.5	4.7	0.8	6.3	2.7	0.7	0.8	NA
D,E	0.0	NA	0.2	0.6	0.3	1.0	0.3	0.0	0.4	NA
R,H,K	1.2	NA	0.3	0.1	0.7	0.4	0.2	0.0	0.2	NA
L,V,I,M	3.0	1.0	10.2	1.0	1.3	2.1	1.4	4.7	0.8	1.0
Y,F,W	+++	NA	2.6	3.1	3.6	0.6	1.6	14.1	2.1	NA
Q,N	1.0	NA	0.9	0.8	0.8	0.8	0.6	0.4	0.7	NA
S,T,C	0.9	NA	0.9	1.1	1.0	0.9	1.4	1.3	2.9	NA
P	0.0	NA	0.4	2.6	0.0	1.0	0.4	1.9	1.2	NA

+++ Indicates that there were no negative binders.

TABLE 13  
Summary of A2.1 Motif-Library 10-mers

AA position	(+)	(-)
1	(YFW), A	(DE), P
2	Anchor	
3	(LVIM)	(DE)
4	G	A, (RKH)
5		P
6	G	
7		(RKH)
8	(YFW), (LVIM)	(DE), (RKH)
9		(RKH)
10	Anchor	

(+) = Ratio  $\geq$  4-fold      (-) = Ratio  $\leq$  0.25

TABLE 14  
A2.1 MOTIF FOR 10-MER PEPTIDES

AA Position	(+)	(-)
1		acidic amino-acids and P
2	Anchor: L, M, (I, V, A, T)	
3		acidic amino-acids
4		basic amino-acids and A
5		P
6		
7		basic amino-acids
8		acidic and basic amino- acids
9		basic amino-acids
10	Anchor: V, I, L (A, M)	

TABLE 15

## COMPARISON OF A2.1 BINDING OF 9-MERS AND 10-MERS

AA Position	9-mers	10-mers
	(+)	(+)
1	(YFW)	(YFW)
2	Anchor	Anchor
3	(YWF)	(LVIM)
4	(STC)	G
5	(YWF)	
6		G
7	A	
8		(YWF), (LVIM)
9	Anchor	
10	-	Anchor

AA Position	9-mers	10-mers
	(-)	(-)
1	P, (DE)	P, (DE)
2	Anchor	Anchor
3	(DE), (RKH)	(DE)
4		A, (RKH)
5		P
6	(RKH)	
7	(DE), (RKH)	(RKH)
8		(DE), (RKH)
9	Anchor	(RKH)
10	-	Anchor

were preferred in 9-mers, aliphatic residues (L, V, I, M) were preferred in 10-mers.

At the C-terminus of the peptides, basic amino acids are not favored in position 7, and both acidic and basic amino acids are not favored in position 8 for 10-mers. This is in striking agreement with the observation that the same pattern was found in 9-mers at positions 6 and 7. Interestingly, again the favored residues differ between two peptides sizes. Aromatic (Y, F, W) or aliphatic (L, V, I, M) residues were preferred in 10-mers at position 8, while the A residue was preferred by 9-mers in the corresponding position 7.

By contrast, in the center of the peptide no similarities of frequency preferences were observed at positions 4, 5, and 6 in 10-mers and positions 4 and 5 in the 9-mers.

Most interestingly, among the residues most favored in the center of the tested peptides were G in position 4 and 6, P in position 5 was not observed in binders. All of these residues are known to dramatically influence the overall secondary structure of peptides, and in particular would be predicted to strongly influence the propensity of a 10-mer to adopt a "kinked" or "bulged" conformation.

Charged residues are predominantly deleterious for binding and are frequently observed in non-binders of 9-mers and 10-mers.

However, favored residues are different for 9-mers and 10-mers. Glycine is favored while Proline is disfavored in the center of 10-mer peptides but this is not the case for 9-mers.

These data establish the existence of an "insertion area" spanning two positions (4, 5) in 9-mers and 3 positions (4, 5, 6) in 10-mers. This insertion area is a more permissive region where few residue similarities are observed between the 9-mer and 10-mer antigenic peptides. Furthermore, in addition to the highly conserved anchor positions 2 and 9, there are "anchor areas" for unfavored residues in positions 1 and 3 at the N-terminus for both 9-mer and 10-mer and

positions 7-10 or 6-9 at the C-terminus for 10-mers and 9-mers, respectively.

#### Example 6

##### 5     Algorithm to Predict Binding of 9-mer Peptides to HLA-A2.1

Within the population of potential A2.1 binding peptides identified by the 2,9 motif, as shown in the previous example, only a few peptides are actually good or intermediate binders and thus potentially immunogenic. It is apparent from  
10 the data previously described that the residues present in positions other than 2 and 9 can influence, often profoundly, the binding affinity of a peptide. For example, acidic residues at position 1 for A2.1 peptides do not appear to be tolerated. Therefore, a more exact predictor of binding could  
15 be generated by taking into account the effects of different residues at each position of a peptide sequence, in addition to positions 2 and 9.

More specifically, we have utilized the data bank obtained during the screening of our collection of A2.1 motif  
20 containing 9-mer peptides to develop an algorithm which assigns a score for each amino acid, at each position along a peptide. The score for each residue is taken as the ratio of the frequency of that residue in good and intermediate binders to the frequency of occurrence of that residue in non-binders.

25     In the present "Grouped Ratio" algorithm residues have been grouped by similarity. This avoids the problem encountered with some rare residues, such as tryptophan, where there are too few occurrences to obtain a statistically significant ratio. Table 16 is a listing of scores obtained  
30 by grouping for each of the twenty amino acids by position for 9-mer peptides containing perfect 2/9 motifs. A peptide is scored in the "Grouped Ratio" algorithm as a product of the scores of each of its residues. In the case of positions other than 2 and 9, the scores have been derived using a set  
35 of peptides which contain only preferred residues in positions 2 and 9. To enable us to extend our "Grouped Ratio" algorithm



TABLE 16

	1	2	3	4	5	6	7	8	9
A	2.6	0.03	0.87	0.87	0.65	0.87	4.4	0.29	0.16
C	0.73	0.01	1.9	4.8	0.87	1.2	1.2	1.1	0.01
D	0.10	0.01	0.10	0.65	0.29	0.65	0.11	0.87	0.01
E	0.10	0.01	0.10	0.65	0.29	0.65	0.11	0.87	0.01
F	7.0	0.01	5.2	0.87	8.7	2.0	2.3	2.6	0.01
G	3.5	0.01	0.44	1.1	1.1	1.3	0.44	0.44	0.01
H	3.1	0.01	0.22	1.0	0.87	0.09	0.10	1.3	0.01
I	3.1	0.14	1.8	0.55	0.87	1.4	1.2	1.8	0.40
K	3.1	0.01	0.22	1.0	0.87	0.09	0.10	1.3	0.01
L	3.1	1.00	1.8	0.55	0.87	1.4	1.2	1.8	0.09
M	3.1	2.00	1.8	0.55	0.87	1.4	1.2	1.8	0.06
N	0.50	0.01	0.37	1.2	0.87	1.1	0.65	0.33	0.01
P	0.12	0.01	0.70	0.73	2.6	1.8	2.9	0.10	0.01
Q	0.50	0.01	0.37	1.2	0.87	1.1	0.65	0.33	0.01
R	3.1	0.01	0.22	1.0	0.87	0.09	0.10	1.3	0.01
S	0.73	0.01	1.9	4.8	0.87	1.2	1.2	1.1	0.01
T	0.73	0.01	1.9	4.8	0.87	1.2	1.2	1.1	0.01
V	3.1	0.08	1.8	0.55	0.87	1.4	1.2	1.8	1.00
W	7.0	0.01	5.2	0.87	8.7	2.0	2.3	2.6	0.01
Y	7.0	0.01	5.2	0.87	8.7	2.0	2.3	2.6	0.01

to peptides which may have residues other than the preferred ones at 2 and 9, scores for 2 and 9 have been derived from a set of peptides which are single amino acid substitutions at positions 2 and 9. Figure 2 shows a scattergram of the log of relative binding plotted against "Grouped Ratio" algorithm score for our collection of 9-mer peptides from the previous example.

The present "Grouped Ratio" algorithm can be used to predict a population of peptides with the highest occurrence of good binders. If one were to rely, for example, solely on a 2(L,M) and 9(V) motif for predicting A2.1 binding 9-mer peptides, it would have been predicted that all 160 peptides in our database would be good binders. In fact, as has already been described, only 12% of these peptides would be described as good binders and only 22% as intermediate binders; 66% of the peptides predicted by such a 2,9 motif are either weak or non-binding peptides. In contrast, using the "Grouped Ratio" algorithm described above, and selecting a score of 1.0 as threshold, 41 peptides were selected. Of this set, 27% are good binders, and 49% are intermediate, while only 20% are weak and 5% are non-binders (Table 17).

The present example of an algorithm has used the ratio of binders/non-binders to measure the impact of a particular residue at each position of a peptide. It is immediately apparent to one of ordinary skill that there are alternative ways of creating a similar algorithm.

An algorithm using the average binding affinity of all the peptides with a certain amino acid (or amino acid type) at a certain position has the advantage of including all of the peptides in the analysis, and not just good/intermediate binders and non-binders. Moreover, it gives a more quantitative measure of affinity than the simpler "Grouped Ratio" algorithm. We have created such an algorithm by calculating for each amino acid, by position, the average log of binding when that particular residue occurs in our set of 160 2,9 motif containing peptides. These values are shown in Table 18. The algorithm score for a peptide is then taken as the sum of the scores by position for each residues.

Figure 3 shows a scattergram of the log of relative binding against the average "Log of Binding" algorithm score. Table 17 shows the ability of the two algorithms to predict peptide binding at various levels, as a function of the cut-off score used. The ability of a 2,9 motif to predict binding in the same peptide set is also shown for reference purposes. It is clear from this comparison that both algorithms of this invention have a greater ability to predict populations with higher frequencies of good binders than a 2,9 motif alone. Differences between the "Grouped Ratio" algorithm and the "Log of Binding" algorithm are small in the set of peptides analyzed here, but do suggest that the "Log of Binding" algorithm is a better, if only slightly, predictor than the "Grouped Ratio" algorithm.

The log of binding algorithm was further revised in two ways. First, poly-alanine (poly-A) data were incorporated into the algorithms at the anchor positions for residues included in the expanded motifs where data obtained by screening a large library of peptides were not available. Second, an "anchor requirement screening filter" was incorporated into the algorithm. The poly-A approach is described in detail, above. The "anchor requirement screening filter" refers to the way in which residues are scored at the anchor positions, thereby providing the ability to screen out peptides which do not have preferred or tolerated residues in the anchor positions. This is accomplished by assigning a score for unacceptable residues at the anchor positions which are so high as to preclude any peptide which contains them from achieving an overall score which would allow it to be considered as a potential binder.

The results for 9-mers and 10-mers are presented in Tables 26 and 27, below. In these tables, values are group values as follows: A; G; P; D,E; R,H,K; L,I,V,M; F,Y,W; S,T,C; and Q,N, except where noted in the tables.

TABLE 17

Criteria	Cut-off	Good Binders	Intermediate Binders	Weak Binders	Negative Binders	Totals
2.9 motif		19 (12%)	36 (22%)	58 (36%)	48 (30%)	161 (100%)
<b>Grouped Ratio</b>						
Algorithm	1.5	5 (83%)	1 (17%)	0 (0%)	0 (0%)	6 (100%)
	1.25	8 (67%)	4 (33%)	0 (0%)	0 (0%)	12 (100%)
	1	10 (50%)	9 (45%)	1 (5%)	0 (0%)	20 (100%)
	0.5	12 (32%)	17 (46%)	7 (19%)	1 (3%)	37 (100%)
	0	12 (23%)	26 (49%)	12 (23%)	3 (6%)	53 (100%)
	-1	17 (18%)	35 (37%)	33 (35%)	10 (11%)	95 (100%)
	-2	19 (15%)	36 (29%)	50 (40%)	21 (17%)	126 (100%)
	-3	19 (13%)	36 (24%)	56 (38%)	38 (26%)	149 (100%)
	no cut	19 (12%)	36 (22%)	58 (36%)	48 (30%)	161 (100%)
<b>Log of Binding</b>						
Algorithm	-19	5 (100%)	0 (0%)	0 (0%)	0 (0%)	5 (100%)
	-20	8 (73%)	3 (27%)	0 (0%)	0 (0%)	11 (100%)
	-21	15 (43%)	15 (43%)	5 (14%)	0 (0%)	35 (100%)
	-22	17 (26%)	27 (41%)	21 (32%)	1 (2%)	68 (100%)
	-23	18 (19%)	35 (37%)	34 (36%)	7 (7%)	94 (100%)
	-24	18 (16%)	36 (30%)	47 (39%)	17 (14%)	119 (100%)
	-25	19 (14%)	36 (26%)	55 (39%)	30 (21%)	140 (100%)
	no cut	19 (12%)	36 (22%)	58 (36%)	48 (30%)	161 (100%)

TABLE 18

	1	2	3	4	5	6	7	8	9
A	-2.38	-3.22	-2.80	-2.68	-2.89	-2.70	-2.35	-3.07	-2.49
C	-2.94	-4.00	-2.58	-1.96	-3.29	-2.22	-2.97	-2.37	-4.00
D	-3.69	-4.00	-3.46	-2.71	-2.26	-2.63	-3.61	-3.03	-4.00
E	-3.64	-4.00	-3.51	-2.65	-3.39	-3.41	-3.21	-2.63	-4.00
F	-1.89	-4.00	-2.35	-2.50	-1.34	-2.43	-2.18	-1.71	-4.00
G	-2.32	-4.00	-3.04	-2.63	-2.56	-2.30	-3.13	-2.96	-4.00
H	-2.67	-4.00	-2.58	-2.58	-2.05	-3.32	-3.13	-2.16	-4.00
I	-1.65	-2.55	-2.80	-3.44	-2.74	-2.79	-2.20	-2.69	-2.10
K	-2.51	-4.00	-3.65	-2.93	-3.34	-3.77	-3.13	-3.27	-4.00
L	-2.32	-1.70	-2.02	-2.49	-2.71	-2.63	-2.62	-2.01	-2.74
M	-0.39	-1.39	-1.79	-3.07	-3.43	-1.38	-1.33	-0.97	-2.96
N	-3.12	-4.00	-3.52	-2.22	-2.36	-2.30	-3.14	-3.31	-4.00
P	-3.61	-4.00	-2.97	-2.64	-2.42	-2.31	-1.83	-2.42	-4.00
Q	-2.76	-4.00	-2.81	-2.63	-3.06	-2.84	-2.12	-3.05	-4.00
R	-1.92	-4.00	-3.41	-2.61	-3.05	-3.76	-3.43	-3.02	-4.00
S	-2.39	-3.52	-2.04	-2.12	-2.83	-3.04	-2.73	-2.02	-4.00
T	-2.92	-4.00	-2.60	-2.48	-2.17	-2.58	-2.67	-3.14	-3.70
V	-2.44	-2.64	-2.68	-3.29	-2.49	-2.24	-2.68	-2.83	-1.70
W	-0.14	-4.00	-1.01	-2.94	-1.63	-2.77	-2.85	-2.13	-4.00
X	-1.99	-2.13	-2.41	-2.97	-2.72	-2.70	-2.41	-2.35	-2.42
Y	-1.46	-4.00	-1.67	-2.70	-1.92	-2.39	-1.35	-3.37	-4.00

Example 7Use of an Algorithm to Predict Binding of 10-mer Peptides to  
HLA-A2.1

5           Using the methods described in the proceeding  
example, an analogous set of algorithms has been developed for  
predicting the binding of 10-mer peptides. Table 19 shows the  
scores used in a "Grouped Ratio" algorithm, and Table 20 shows  
the "Log of Binding" algorithm scores, for 10-mer peptides.  
10 Table 21 shows a comparison of the application of the two  
different algorithmic methods for selecting binding peptides.  
Figures 4 and 5 show, respectively, scattergrams of a set of  
10-mer peptides containing preferred residues in positions 2  
and 10 as scored by the "Grouped Ratio" and "Log of Binding"  
15 algorithms.

TABLE 19

	1	2	3	4	5	6	7	8	9	10
A	3.00	0.01	3.10	0.20	1.60	0.60	1.30	1.60	0.50	0.01
C	0.90	0.01	0.90	1.10	1.00	0.90	1.40	1.30	2.90	0.01
D	0.01	0.01	0.20	0.60	0.30	1.00	0.30	0.01	0.40	0.01
E	0.01	0.01	0.20	0.60	0.30	1.00	0.30	0.01	0.40	0.01
F	3.00	0.01	2.60	3.10	3.60	0.60	1.60	14.1	2.10	0.01
G	0.80	0.01	0.50	4.70	0.80	6.30	2.70	0.70	0.80	0.01
H	1.20	0.01	0.30	0.10	0.70	0.40	0.20	0.01	0.20	0.01
I	3.00	0.50	10.2	1.00	1.30	2.10	1.40	4.70	0.80	1.00
K	1.20	0.01	0.30	0.10	0.70	0.40	0.20	0.01	0.20	0.01
L	3.00	1.10	10.2	1.00	1.30	2.10	1.40	4.70	0.80	0.50
M	3.00	0.60	10.2	1.00	1.30	2.10	1.40	4.70	0.80	0.01
N	1.00	0.01	0.90	0.80	0.80	0.80	0.60	0.40	0.70	0.01
P	0.00	0.01	0.40	2.60	0.01	1.00	0.40	1.90	1.20	0.01
Q	1.00	0.01	0.90	0.80	0.80	0.80	0.60	0.40	0.70	0.01
R	1.20	0.01	0.30	0.10	0.70	0.40	0.20	0.01	0.20	0.01
S	0.90	0.01	0.90	1.10	1.00	0.90	1.40	1.30	2.90	0.01
T	0.90	0.01	0.90	1.10	1.00	0.90	1.40	1.30	2.90	0.01
V	3.00	0.10	10.2	1.00	1.30	2.10	1.40	4.70	0.80	2.30
W	3.00	0.01	2.60	3.10	3.60	0.60	1.60	14.1	2.10	0.01
Y	3.00	0.01	2.60	3.10	3.60	0.60	1.60	14.1	2.10	0.01

TABLE 20

	1	2	3	4	5	6	7	8	9	10
A	-2.40	-4.00	-2.54	-3.42	-3.07	-3.30	-2.98	-2.69	-3.29	-4.00
C	-3.64	-4.00	-2.47	-2.48	-1.78	-3.94	-1.28	-3.10	-2.43	-4.00
D	-3.65	-4.00	-2.76	-3.26	-2.76	-3.03	-3.43	-3.68	-3.63	-4.00
E	-3.92	-4.00	-3.63	-3.34	-3.73	-2.82	-3.54	-3.71	-2.95	-4.00
F	-1.52	-4.00	-1.96	-3.03	-2.01	-3.11	-2.67	-1.61	-2.43	-4.00
G	-2.91	-4.00	-3.40	-2.63	-2.98	-2.45	-2.52	-3.18	-3.03	-4.00
H	-3.61	-4.00	-3.10	-3.03	-2.33	-2.99	-3.70	-3.55	-4.00	-4.00
I	-2.26	-4.00	-2.82	-3.05	-2.38	-2.61	-2.38	-3.34	-3.18	-1.47
K	-2.53	-4.00	-3.65	-3.42	-3.14	-3.58	-3.50	-3.53	-4.00	-4.00
L	-2.00	-2.93	-2.21	-2.48	-2.88	-2.53	-2.57	-1.83	-3.23	-3.20
M	-2.41	-3.11	-2.00	-3.33	-3.70	-2.56	-3.27	-2.25	-3.00	-4.00
N	-3.21	-4.00	-3.09	-2.61	-2.93	-2.89	-3.52	-3.01	-2.88	-4.00
P	-3.90	-4.00	-3.21	-2.27	-3.72	-3.06	-3.35	-2.58	-2.94	-4.00
Q	-2.92	-4.00	-2.97	-4.00	-2.98	-3.46	-2.20	-3.23	-3.45	-4.00
R	-3.01	-4.00	-3.44	-3.50	-3.23	-3.32	-3.72	-3.59	-2.97	-4.00
S	-2.47	-4.00	-3.17	-3.11	-3.23	-2.64	-3.19	-2.79	-2.26	-4.00
T	-3.59	-4.00	-3.07	-2.88	-2.89	-3.16	-2.43	-3.11	-2.58	-4.00
V	-2.97	-4.00	-2.46	-3.14	-3.27	-2.53	-3.14	-3.02	-2.90	-2.61
W	-2.10	-4.00	-2.72	-1.79	-2.65	-1.92	-1.80	-2.24	-2.11	-4.00
Y	-2.37	-4.00	-2.42	-2.85	-3.03	-3.76	-2.82	-2.34	-2.74	-4.00



TABLE 21

Criteria	Cut-off	Good	Intermediate	Weak	Negative	Totals
2,10 motif		10 (6%)	29 (17%)	70 (41%)	61 (36%)	170 (100%)
Grouped Ratio	4	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Algorithm	3	1 (25%)	2 (50%)	1 (25%)	0 (0%)	4 (100%)
	2	6 (24%)	13 (52%)	6 (24%)	0 (0%)	25 (100%)
	1	10 (21%)	21 (45%)	16 (34%)	0 (0%)	47 (100%)
	0	10 (15%)	28 (42%)	26 (39%)	2 (3%)	66 (100%)
	-1	10 (11%)	29 (32%)	42 (46%)	11 (12%)	92 (100%)
	-2	10 (9%)	29 (25%)	54 (47%)	23 (20%)	116 (100%)
	-3	10 (7%)	29 (22%)	63 (47%)	32 (24%)	134 (100%)
	no cut	10 (6%)	29 (17%)	70 (41%)	61 (36%)	170 (100%)
Log of Binding	-24	2 (50%)	2 (50%)	0 (0%)	0 (0%)	4 (100%)
Algorithm	-25	5 (56%)	3 (33%)	1 (11%)	0 (0%)	9 (100%)
	-26	7 (47%)	5 (33%)	3 (20%)	0 (0%)	15 (100%)
	-27	10 (32%)	9 (29%)	12 (39%)	0 (0%)	31 (100%)
	-28	10 (17%)	19 (33%)	29 (50%)	0 (0%)	58 (100%)
	-29	10 (12%)	25 (30%)	48 (58%)	0 (0%)	83 (100%)
	-30	10 (10%)	29 (28%)	59 (57%)	5 (5%)	103 (100%)
	-31	10 (8%)	28 (22%)	66 (51%)	24 (19%)	129 (100%)
	-32	10 (7%)	29 (19%)	70 (47%)	40 (27%)	149 (100%)
	no cut	10 (6%)	29 (17%)	70 (41%)	61 (36%)	170 (100%)

Example 8Binding of A2.1 Algorithm Predicted Peptides

The results of Examples 6 and 7 indicate that an algorithm can  
5 be used to select peptides that bind to HLA-A2.1 sufficiently  
to have a high probability of being immunogenic.

To test this result, we tested our algorithm on a large (over  
1300) non-redundant, independent set of peptides derived from  
various sources. After scoring this set with our algorithm,  
10 we selected 41 peptides (Table 21) for synthesis, and tested  
them for A2.1 binding. This set of peptides was comprised of  
21 peptides with high algorithm scores, and 20 peptides with  
low algorithm scores.

The binding data and categorization profile are shown in  
15 Tables 22 and 23 respectively. The correlation between  
binding and algorithm score was 0.69. It is immediately  
apparent from Table 23 the striking difference between  
peptides with high algorithm scores, and those with low  
algorithm scores. Respectively, 76% of the high scorers and  
20 none of the low scorers were either good or intermediate  
binders. This data demonstrates the utility of the algorithm  
of this invention.

TABLE 22

SEQUENCE	SOURCE	A2.1 Binding	Algorithm Score
MMWFVVLTV	CMV	0.76	346
YLLLYFSPV	CMV	0.75	312
YLYRLNFCL	CMV	0.72	169
FMWTYLVTL	CMV	0.68	336
LLWWITILL	CMV	0.49	356
GLWCVLFFV	CMV	0.47	1989
LMIRGVLEV	CMV	0.45	296
LLLCRLPFL	CMV	0.42	1356
RLLTSLFFL	HSV	0.34	859
LLLYDYDSL	HSV	0.28	390
AMSRNLFRV	CMV	0.15	1746
AMLTACVEV	CMV	0.089	411
RLQPNVPLV	CMV	0.048	392
VLARTFTPV	CMV	0.044	196
RLLRGURL	CMV	0.037	494
WMWFPSVLL	CMV	0.036	362
YLCCGITLL	CMV	0.021	1043
DMLGRVFFV	HSV	0.011	1422
ALGRYQQLV	CMV	0.0089	184
LMPPPVAEL	CMV	0.0066	416
LMCRYTPRL	CMV	0.0055	414
RLTWRLTWL	CMV	0.0052	250
AMPRRVLHV	CMV	0.0014	628
ALLLVLALL	CMV	0.0014	535
AMSGTGTTL	CMV	0.0005	602
MLNVMKEAV	CMV	0.0039	0.00031
TMELMIRTV	CMV	0.0029	0.0013
TLAAMHSKL	HSV	0.0008	0.0019
TLNIVRDHV	CMV	0.0005	0.00021
ELSIFRERL	HSV	0.0002	0.0020
FLRVQOKAL	HSV	0.0002	0.00099
ELQMMQDWV	CMV	0.0001	0.0020
QLNAMKPD	MT	0.0001	0.0017
GLRQLKGAL	CMV	0.0001	0.0010
TLRMSSKAV	HSV	0.0001	0.00085
SLRIKRELL	CMV	0	0.00041
DLKQMERVV	CMV	0	0.00026
PLRVTPSDL	CMV	0	0.0019
QLDYEKQVL	CMV	0	0.0012
WLKLLRDAL	CMV	0	0.0012
PMEAVRHPL	CMV	0	0.0011
ELKQTRVNL	CMV	0	0.00053
NLEVIHDAL	CMV	0	0.00050
ELKKVKSVL	HSV	0	0.00033
PLAYERDKL	CMV	0	0.00017

TABLE 23

Set	Good Binders	Intermediate Binders	Weak Binders	Negative Binders	Totals
HI Scorers	11 (52.4%)	5 (23.8%)	5 (23.8%)	0 (0.0%)	21 (100%)
Low Scorers	0 (0.0%)	C (0.0%)	10 (50.0%)	10 (50.0%)	20 (100%)
Totals	11 (26.6%)	5 (12.2%)	15 (36.6%)	10 (24.4%)	41 (100%)

## Example 9

Ex vivo induction of Cytotoxic T Lymphocytes (CTL)

Peripheral blood mononuclear cells (PBMC) are isolated from an HLA-typed patient by either venipuncture or apheresis (depending upon the initial amount of CTLp required), and purified by gradient centrifugation using Ficoll-Paque (Pharmacia). Typically, one can obtain one million PBMC for every ml of peripheral blood, or alternatively, a typical apheresis procedure can yield up to a total of  $1-10 \times 10^{10}$  PBMC.

The isolated and purified PBMC are co-cultured with an appropriate number of antigen presenting cell (APC), previously incubated ("pulsed") with an appropriate amount of synthetic peptide (containing the HLA binding motif and the sequence of the antigen in question). PBMC are usually incubated at  $1-2 \times 10^6$  cells/ml in culture medium such as RPMI-1640 (with autologous serum or plasma) or the serum-free medium AIM-V (Gibco).

APC are usually used at concentrations ranging from  $1 \times 10^4$  to  $2 \times 10^5$  cells/ml, depending on the type of cell used. Possible sources of APC include: 1) autologous dendritic cells (DC), which are isolated from PBMC and purified as described (Inaba, et al., J. Exp. Med. 166:182 (1987)); and 2) mutant and genetically engineered mammalian cells that express "empty" HLA molecules (which are syngeneic [genetically identical] to the patient's allelic HLA form), such as the, mouse RMA-S cell line or the human T2 cell line. APC containing empty HLA molecules are known to be potent inducers of CTL responses, possibly because the peptide can associate more readily with empty MHC molecules than with MHC molecules which are occupied by other peptides (DeBruijn, et al., Eur. J. Immunol. 21:2963-2970 (1991)).

In those cases when the APC used are not autologous, the cells will have to be gamma irradiated with an appropriate dose (using, e.g., radioactive cesium or cobalt) to prevent their proliferation both ex vivo, and when the cells are re-introduced into the patients.

The mixture cultures, containing PBMC, APC and peptide are kept in an appropriate culture vessel such as plastic T-flasks, gas-permeable plastic bags, or roller bottles, at 37° centigrade in a humid air/CO<sub>2</sub> incubator.

5 After the activation phase of the culture, which usually occurs during the first 3-5 days, the resulting effector CTL can be further expanded, by the addition of recombinant DNA-derived growth factors such as interleukin-2 (IL-2), interleukin-4 (IL-4), or interleukin-7 (IL-7) to the cultures.  
10 An expansion culture can be kept for an additional 5 to 12 days, depending on the numbers of effector CTL required for a particular patient. In addition, expansion cultures may be performed using hollow fiber artificial capillary systems (Cellco), where larger numbers of cells (up to  $1 \times 10^{11}$ ) can be  
15 maintained.

Before the cells are infused into the patient, they are tested for activity, viability, toxicity and sterility. The cytotoxic activity of the resulting CTL can be determined by a standard <sup>51</sup>Cr-release assay (Biddison, W.E. 1991, Current  
20 Protocols in Immunology, p7,17.1-7.17.5, Ed. J. Coligan et al., J. Wiley and Sons, New York), using target cells that express the appropriate HLA molecule, in the presence and absence of the immunogenic peptide. Viability is determined by the exclusion of trypan blue dye by live cells. Cells are  
25 tested for the presence of endotoxin by conventional techniques. Finally, the presence of bacterial or fungal contamination is determined by appropriate microbiological methods (chocolate agar, etc.). Once the cells pass all quality control and safety tests, they are washed and placed  
30 in the appropriate infusion solution (Ringer/glucose lactate) and infused intravenously into the patient.

#### Example 10

##### Assays for CTL Activity

35 1. Peptide synthesis: Peptide syntheses were carried out by sequential coupling of N- $\alpha$ -Fmoc-protected amino acids on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer using standard Fmoc coupling cycles (software

version 1.40). All amino acids, reagents, and resins were obtained from Applied Biosystems or Bachem. Solvents were obtained from Burdick & Jackson. Solid-phase synthesis was started from an appropriately substituted Fmoc-amino acid-Sasrin resin. The loading of the starting resin was 0.5-0.7 mmol/g polystyrene, and 0.1 or 0.25 meq were used in each synthesis. A typical reaction cycle proceeded as follows: 1) The N-terminal Fmoc group was removed with 25% piperidine in dimethylformamide (DMF) for 5 minutes, followed by another treatment with 25% piperidine in DMF for 15 minutes. The resin was washed 5 times with DMF. An N-methylpyrrolidone (NMP) solution of a 4 to 10 fold excess of a pre-formed 1-hydroxybenzotriazole ester of the appropriate Fmoc-amino acid was added to the resin and the mixture was allowed to react for 30-90 min. The resin was washed with DMF in preparation for the next elongation cycle. The fully protected, resin bound peptide was subjected to a piperidine cycle to remove the terminal Fmoc group. The product was washed with dichloromethane and dried. The resin was then treated with trifluoroacetic acid in the presence of appropriate scavengers [e.g. 5% (v/v) water] for 60 minutes at 20°C. After evaporation of excess trifluoroacetic acid, the crude peptide was washed with dimethyl ether, dissolved in water and lyophilized. The peptides were purified to >95% homogeneity by reverse-phase HPLC using H<sub>2</sub>O/CH<sub>3</sub>CN gradients containing 0.2% TFA modifier on a Vydac, 300Å pore-size, C-18 preparative column. The purity of the synthetic peptides was assayed on an analytical reverse-phase column, and their composition ascertained by amino acid analysis and/or sequencing. Peptides were routinely dissolved in DMSO at the concentration of 20 mg/ml.

2. Media: RPMI-1640 containing 10% fetal calf serum (FCS) 2 mM Glutamine, 50 µg/ml Gentamicin and 5x10<sup>-5</sup>M 2-mercaptoethanol served as culture medium and will be referred to as R10 medium.

RPMI-1640 containing 25 mM Hepes buffer and supplemented with 2% FCS was used as cell washing medium.

3.       Rat Concanavalin A supernatant: The spleen cells obtained from Lewis rats (Sprague-Dawley) were resuspended at a concentration of  $5 \times 10^6$  cells/ml in R10 medium supplemented with 5  $\mu\text{g/ml}$  of ConA in 75  $\text{cm}^2$  tissue culture flasks. After  
5 48 hr at  $37^\circ\text{C}$ , the supernatants were collected, supplemented with 1%  $\alpha$ -methyl-D-mannoside and filter sterilized (.45  $\mu\text{m}$  filter). Aliquots were stored frozen at  $-20^\circ\text{C}$ .
4.       LPS-activated lymphoblasts: Murine splenocytes were resuspended at a concentration of  $1-1.5 \times 10^6/\text{ml}$  in R10 medium  
10 supplemented with 25  $\mu\text{g/ml}$  LPS and 7  $\mu\text{g/ml}$  dextran sulfate in 75  $\text{cm}^2$  tissue culture flasks. After 72 hours at  $37^\circ\text{C}$ , the lymphoblasts were collected for use by centrifugation.
5.       Peptide coating of lymphoblasts: Coating of the LPS activated lymphoblasts was achieved by incubating  $30 \times 10^6$   
15 lymphoblasts with 100  $\mu\text{g}$  of peptide in 1 ml of R10 medium for 1 hr at  $37^\circ\text{C}$ . Cells were then washed once and resuspended in R10 medium at the desired concentration for use in in vitro CTL activation.
6.       Peptide coating of Jurkat A2/ $K^b$  cells: Peptide  
20 coating was achieved by incubating  $10 \times 10^6$  irradiated (20,000 rads) Jurkat A2.1/ $K^b$  cells with 20  $\mu\text{g}$  of peptide in 1 ml of R10 medium for 1 hour at  $37^\circ\text{C}$ . Cells were washed three times and resuspended at the required concentration in R10 medium.
7.       In Vitro CTL activation: One to four weeks after  
25 priming spleen cells ( $5 \times 10^6$  cells/well or  $30 \times 10^6$  cells/T25 flask) were concultured at  $37^\circ\text{C}$  with syngeneic, irradiated (3,000 rads), peptide coated lymphoblasts ( $2 \times 10^6$  cells/well or  $10 \times 10^6$  cells/T25 flask) in R10 medium to give a final volume of 2 ml in 24-well plates or 10 ml in T25 flasks.
8.       Restimulation of effector cells: Seven to ten days  
30 after the initial in vitro activation, described in paragraph 7 above, a portion of the effector cells were restimulated with irradiated (20,000 rads), peptide-coated Jurkat A2/ $K^b$  cells ( $0.2 \times 10^6$  cells/well) in the presence of  $3 \times 10^6$  "feeder  
35 cells"/well (C57Bl/6 irradiated spleen cells) in R10 medium supplemented with 5% rat ConA supernatant to help provide all of the cytokines needed for optimal effector cell growth.



9.       Assay for cytotoxic activity: Target cells ( $3 \times 10^6$ ) were incubated at  $37^\circ\text{C}$  in the presence of  $200 \mu\text{l}$  of sodium  $^{51}\text{Cr}$  chromate. After 60 minutes, cells were washed three times and resuspended in R10 medium. Peptides were added at the required concentration. For the assay,  $10^4$   $^{51}\text{Cr}$ -labeled target cells were added to different concentrations of effector cells (final volume of  $200 \mu\text{l}$ ) in U-bottom 96-2311 plates. After a 6-hour incubation period at  $37^\circ\text{C}$ ,  $0.1 \text{ ml}$  aliquots of supernatant were removed from each well and radioactivity was determined in a Micromedic automatic gamma counter. The percent specific lysis was determined by the formula: percent specific release =  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ . Where peptide titrations were performed, the antigenicity of a given peptide (for comparison purposes) was expressed as the peptide concentration required to induce 40% specific  $^{51}\text{Cr}$  release at a given E:T.

Transgenic mice were injected subcutaneously in the base of the tail with an incomplete Freund's adjuvant emulsion containing  $50 \text{ nM}$  of the putative CTL epitopes containing the A2.1 motifs, and  $50 \text{ nM}$  of a hepatitis B core T helper epitope. Eight to 20 days later, animals were sacrificed and spleen cells were restimulated in vitro with syngeneic LPS lymphoblasts coated with the putative CTL epitope. A source of IL-2 (rat con A supernatant) was added at day 6 of the assay to a final concentration of 5% and CTL activity was measured on day 7. The capacity of these effector T cells to lyse peptide-coated target cells that express the A2 KB molecule (Jurkat A2 KB) was measured as lytic units. The results are presented in Table 24.

The results of this experiment indicate that those peptides having a binding of at least 0.01 are capable of inducing CTL. All of the peptides in Appendices 1 and 2 having a binding of at least about 0.01 would be immunogenic.

35

TABLE 24  
Binding and Immunogenicity  
HBV Polymerase (ayw)

Peptide	Binding**	CTL Activity	Algorithm
1 2 3 4 5 6 7 8 9			
F L L S L G I H L	0.52	63	-20.8
G L Y S S T V P V	0.15	10	-21.9
H L Y S H P I I L	0.13	10	-21.1
W I L R G T S F V	0.018	-+	-20.9
N L S W L S L D V	0.013	6	-24.7
L L S S N L S W L	0.005	-	-21.7
N L Q S L T N L L	0.003	-	-23.9
H L L V G S S G L	0.002	-	-24.7
L L D D E A G P L	0.0002	-	-25.5
P L E E E L P R L	0.0001	-	-26.1
D L N L G N L N V	-*	-	-25.7
N L Y V S L L L L	-	-	-23.6
P L P I H T A E L	-	-	-25.04

\*-= $<0.0001$

\*\* Relative binding capacity compared to std with  $IC_{50} = 52mM$   
xxx Lytic units/ $10^6$  cells; 1 lytic unit = the number of  
effector cells required to give 30%  $Cr^{51}$  release.

-, -+ no measurable cytotoxic activity.

Example 11Identification of immunogenic peptides

Using the motifs identified above for HLA-A2.1 allele amino acid sequences from a tumor-related protein, Melanoma Antigen-1 (MAGE-1), were analyzed for the presence of these motifs. Sequences for the target antigen are obtained from the GenBank data base (Release No. 71.0; 3/92). The identification of motifs is done using the "FINDPATTERNS" program (Devereux et al., Nucleic Acids Research 12:387-395 (1984)).

Other viral and tumor-related proteins can also be analyzed for the presence of these motifs. The amino acid sequence or the nucleotide sequence encoding products is obtained from the GenBank database in the cases of Human Papilloma Virus (HPV), Prostate Specific antigen (PSA), p53 oncogene, Epstein Barr Nuclear Antigen-1 (EBNA-1), and c-erb2 oncogene (also called HER-2/neu).

In the cases of Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), and Human Immunodeficiency Virus (HIV) several strains/isolates exist and many sequences have been placed in GenBank.

For HBV, binding motifs are identified for the adr, adw and ayw types. In order to avoid replication of identical sequences, all of the adr motifs and only those motifs from adw and ayw that are not present in adr are added to the list of peptides.

In the case of HCV, a consensus sequence from residue 1 to residue 782 is derived from 9 viral isolates. Motifs are identified on those regions that have no or very little (one residue) variation between the 9 isolates. The sequences of residues 783 to 3010 from 5 viral isolates were also analyzed. Motifs common to all the isolates are identified and added to the peptide list.

Finally, a consensus sequence for HIV type 1 for North American viral isolates (10-12 viruses) was obtained from the Los Alamos National Laboratory database (May 1991 release) and analyzed in order to identify motifs that are constant throughout most viral isolates. Motifs that bear a

small degree of variation (one residue, in 2 forms) were also added to the peptide list.

Appendices 1 and 2 provide the results of searches of the following antigens cERB2, EBNA1, HBV, HCV, HIV, HPV, MAGE, p53, and PSA. Only peptides with binding affinity of at least 1% as compared to the standard peptide in assays described in Example 5 are presented. Binding as compared to the standard peptide is shown in the far right column. The column labeled "Pos." indicates the position in the antigenic protein at which the sequence occurs.

#### Example 12

##### Identification of immunogenic peptides

Using the motifs disclosed here, amino acid sequences from various antigens were screened for further motifs. Screening was carried out as described in Example 11. Tables 25 and 26 provide the results of searches of the following antigens cERB2, CMV, Influenza A, HBV, HIV, HPV, MAGE, p53, PSA, Hu S3 ribosomal protein, LCMV, and PAP. Only peptides with binding affinity of at least 1% as compared to the standard peptide in assays described in Example 5 are presented. Binding as compared to the standard peptide is shown for each peptide.

TABLE 25

Sequence	Antigen	Molecule	A2 Bind.
KIFGSLAFL	c-ERB2		0.1500
RILHNGAYSL	c-ERB2		0.0180
IISAVVGILL	c-ERB2		0.0120
MMWFVVLTV	CMV		0.7600
YLLLYFSPV	CMV		0.7500
YLYRLNFCL	CMV		0.7200
FMWTYLVTL	CMV		0.6800
LLWWITILL	CMV		0.4900
GLWCVLFFV	CMV		0.4700
LMIRGVLEV	CMV		0.4500
LLLCRLPFL	CMV		0.4200
AMSRNLFVR	CMV		0.1500
AMLTACVEV	CMV		0.1000
RIQPNVPLV	CMV		0.0480
VLARTFTPV	CMV		0.0440
RLLRGLIRL	CMV		0.0370
WMWFPSVLL	CMV		0.0360
YLCCGITLL	CMV		0.0210
SLLTEVETYV	FLU-A	M1	0.0650
LLTEVETYV	FLU-A	M1	0.2000
LLTEVETYVL	FLU-A	M1	0.0130
GILGFVFTL	FLU-A	M1	0.1900
GILGFVFTLT	FLU-A	M1	0.0150
ILGFVFTLT	FLU-A	M1	0.2600
ILGFVFTLTV	FLU-A	M1	0.0550
ALASCMGLI	FLU-A	M1	0.0110
RMGAVTTEV	FLU-A	M1	0.0200

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
VTTEVAFGL	FLU-A	M1	0.0360
MVTTTNPLI	FLU-A	M1	0.0150
FTFSPTYKA	HBV	POL	0.0190
YLHTLWKAGI	HBV	POL	0.0280
LMLQAGFFLV	HBV (a)	ENV (a)	0.6300
RMLTIPQSV	HBV (a)	ENV (a)	0.0580
SLDSWWTSTV	HBV (a)	ENV (a)	0.1000
FMLLLCLIFL	HBV (a)	ENV (a)	0.0450
LLPFVQWFFV	HBV (a)	ENV (a)	0.6500
LMPFVQWFFV	HBV (a)	ENV (a)	0.8300
FLGLSPTVWV	HBV (a)	ENV (a)	0.0300
SMLSPFLPLV	HBV (a)	ENV (a)	0.9700
GLWIRTPPV	HBV (a)	ENV (a)	0.3600
NLGNLNVSV	HBV (a)	ENV (a)	0.0160
YLHTLWKAGV	HBV (a)	POL (a)	0.1500
RLTGGVFLV	HBV (a)	POL (a)	0.1600
RMTGGVFLV	HBV (a)	POL (a)	0.1500
RLTGGVFLV	HBV (a)	ENV (a)	0.1600
ILGLLGFAV	HBV (a)	ENV (a)	0.0600
GLCQVFADV	HBV (a)	ENV (a)	0.0300
WLLRGTSFV	HBV (a)	ENV (a)	0.1000
YLPSALNPV	HBV (a)	ENV (a)	0.3200
LLVPFVQWFA	HBV adr		0.2600
FLPSDFFPSI	HBV adr		0.2100
VVSYVNVNM	HBV adr		0.0100
HLPDRVHFA	HBV adr		0.0160
SLAFSAVPA	HBV adr		0.0340

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
FLLTKILTI	HBV adw		0.6300
SLYNILSPFM	HBV adw		0.0440
CLFHIVNLI	HBV adw		0.2100
RLPDRVHFA	HBV adw		0.0940
ALPPASPSA	HBV adw		0.0710
GLLGWSPQA	HBV ayw		0.8650
FLGPLLVLQA	HBV ayw		0.0190
FLLTRILTI	HBV ayw		0.9300
GMLPVCPLI	HBV ayw		0.0520
QLFHLCLII	HBV ayw		0.0390
KLCLGWLWGM	HBV ayw		0.0210
LLWFHISCLI	HBV ayw		0.0130
YLVSEFGVWI	HBV ayw		2.7000
LLEDWGPCA	HBV ayw		0.0180
KLHLYSHPI	HBV ayw		0.2900
FLLAQFTSA	HBV ayw		0.6600
LLAQFTSAI	HBV ayw		9.6000
YMDDVVLGA	HBV ayw		0.1600
ALMPYACI	HBV ayw		0.2000
GLCQVFADA	HBV ayw		0.0180
HLPDLVHFA	HBV ayw		0.1100
RLCCQLDPA	HBV ayw		0.0290
ALMPYACI	HBV ayw polymerase		0.5000
FLCKQYLN	HBV ayw polymerase 665-673		0.0210

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
SLYADSPSV	HBV polymerase		0.3500
ALMPLYASI	HBV polymerase		0.0760
NLNNLNVSI	HBV polymerase		0.0660
ALSLIVNLL	HBV polymerase		0.0470
KLHLYSHPI	HBV polymerase		0.2900
WILRGTSFV	HBV polymerase 1344-1352		0.0270
LVLQAGFFLL	HBVadr	ENV	0.0150
FILLCLIFL	HBVadr	ENV	0.0280
WILRGTSFV	HBVadr	POL	0.0180
IISCTCPTV	HBVadw	PreCore	0.0190
LVPFVQWFV	HBVadw	ENV	0.0200
LIISCSCPTV	HBVadw	CORE	0.0290
FLPSDFFPSI	HBVayr	PreCore	0.2100
LLCLGWLWGM	HBVayr	PreCore	0.0220
QLFHLCLII	HBVayw	PreCore	0.0390
CLGWLTGMDI	HBVayw	PreCore	0.0190
FLGGTTVCL	HBVayw	ENV	0.1700
SLYSILSPFL	HBVayw	ENV	0.2000
FLPSDFFPSV	HBVayw	CORE	1.5000
ILCWGELMTL	HBVayw	CORE	0.1900
LMTLATWVG	HBVayw	CORE	0.6800
TLATWVGVL	HBVayw	CORE	0.5700



Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
GLSRYVARL	HBVayw	POL	0.1200
FLCKQYLNL	HBVayw	POL	0.1700
RMRGTFSAPL	HBVayw	POL	0.0110
SLYADSPSV	HBVayw	POL	0.3500
YLYGVGSAV	HCV		0.1600
LLSTTEWQV	HCV		0.0480
IIGAETFYV	HIV	POL	0.0260
QLWVTVYYGV	HIV	ENV	0.0250
NLWVTVYYGV	HIV	ENV	0.0160
KLWVTVYYGV	HIV	ENV	0.0150
KLWVTVYYGV	HIV.MN gp160		0.0150
YMLDLQPET	HPV16	E7	1.4000
TLGIVCPI	HPV16	E7	0.6500
YLLDLQEPV	HPV16 (a)	E7 (a)	0.2200
YMLDLQPEV	HPV16 (a)	E7 (a)	1.9000
MLDLQPETT	HPV16E7	E7	0.0130
SLQDIEITCVYCKTV	HPV18	E6	0.0100
RLLTSLFFL	HSV		0.3400
RLLTSLFFL	HSV		0.3400
LLLYDYDSL	HSV		0.2800
DMLGRVFFV	HSV		0.0110
TMFEALPHI	LCMV	Gp	0.2000
ALISFLLLA	LCMV	Gp	0.2200
TLMSIVSSL	LCMV	Gp	0.2000
NISGYNFSL	LCMV	Np	0.0280
ALLDGGNML	LCMV	Np	0.0320

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
ALHLFKTTV	LCMV	Gp	0.0170
SLISDQLLM	LCMV	Gp	0.0540
WLVTNQSYL	LCMV	Gp	0.0180
ALMDLLMFS	LCMV	Gp	0.4300
LMDLLMFST	LCMV	Gp	0.0460
LMFSTSAYL	LCMV	Gp	0.3600
YLVSIPLHL	LCMV	Gp	0.4200
SLHCKPEEA	MAGE1		0.0130
ALGLVCVQA	MAGE1		0.0150
LVLGTLEEV	MAGE1		0.0320
GTLEEVPTA	MAGE1		0.0130
CILESIFRA	MAGE1		0.0460
KVADLVGFLL	MAGE1		0.0560
KVADLVGFLLL	MAGE1		0.0200
VMIAEGGHA	MAGE1		0.0360
SMHCKPEEV	MAGE1 (a)		0.0180
AMGLVCVQV	MAGE1 (a)		0.0120
LMLGTLEEV	MAGE1 (a)		0.1300
KMADLVGFLV	MAGE1 (a)		1.5000
VMVTCLGLSV	MAGE1 (a)		0.3000
LLGDNQIMV	MAGE1 (a)		0.0430
QMMPKTGFLV	MAGE1 (a)		0.0500
VMIAEGGHV	MAGE1 (a)		0.0530
WMELSVMEV	MAGE1 (a)		0.0410
FLWGPRALA	MAGE1N		0.0420
RALAETSYV	MAGE1N		0.0100
ALAETSYVKVL	MAGE1N		0.0120

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
ALAETSYVKV	MAGE1N		0.0150
KVLEYVIKV	MAGE1N		0.0900
YVIKVSARV	MAGE1N		0.0140
ALREEEEGV	MAGE1N		0.0210
YMFLWGPRV	MAGE1N(a)		0.2200
KMVELVHFLLL	MAGE2		0.6700
KMVELVHFL	MAGE2		0.1600
KMVELVHFLL	MAGE2		0.1100
KASEYLQLV	MAGE2		0.0110
YLQLVFGIEV	MAGE2		0.3700
LVFGIEVVEV	MAGE2		0.0120
QLVFGIELMEV	MAGE3		0.3400
KVAELVHFL	MAGE3		0.0550
KVAELVHFLL	MAGE3		0.0120
ELMEVDPIGHL	MAGE3		0.0260
HLYIFATCLGL	MAGE3		0.0410
IMPKAGLLIIV	MAGE3		0.0130
LVFGIELMEV	MAGE3		0.1100
ALGRNSFEV	p53 264-272 A8 (A1)		0.0570
LLGANSFEV	p53 264-272 A8 (A4)		0.1100
LLGRASFEV	p53 264-272 A8 (A5)		0.2200
LLGRNAFEV	p53 264-272 A8 (A6)		0.0390
LLGRNSFAV	p53 264-272 A8 (A8)		0.0420
RLGRNSFEV	p53 264-272 A8 (R1)		0.0190
LLGRRSFEV	p53 264-272 A8 (R5)		0.0540
LLGRNSFRV	p53 264-272 A8 (R8)		0.0250
LLFFWLDRSV	PAP		0.6000

Table 25 (Cont'd)

Sequence	Antigen	Molecule	A2 Bind.
VLAKELKFV	PAP		0.0590
ILLWQPIPV	PAP		1.3000
IMYSAHDITTV	PAP		0.0610
FLTLSVTWI	PSA		0.0150
FLTLSVTWIGA	PSA		0.0160
FLTLSVTWI	PSA		0.0150
VLVHPQWVLTA	PSA		0.0130
SLFHPEDTGQV	PSA		0.0190
MLLRLSEPABL	PSA		0.1400
ALGTTCYA	PSA		0.0230
KLQCVDLHVI	PSA		0.0370
FLPSDYFPSV	HBVc18-27 analog		1.0000
YSFLPSDFFPSV	HBVc18-27 analog		0.0190

Table 26

Sequence	Antigen	Molecule	A2 Bind.
ALFLGFLGAA	HIV	gp160	0.4950
MLQLTVWGI	HIV	gp160	0.2450
RVIEVLQRA	HIV	gp160	0.1963
KLTPLCVTL	HIV	gp160	0.1600
LLIAARIVEL	HIV	gp160	0.1550
SLLNATDIAV	HIV	gp160	0.1050
ALFLGFLGA	HIV	gp160	0.0945
HMLQLTVWGI	HIV	gp160	0.0677
LLNATDIAV	HIV	gp160	0.0607
ALLYKLDIV	HIV	gp160	0.0362
WLWYIKIFI	HIV	gp160	0.0355
TIIVHLNESV	HIV	gp160	0.0350
LLQYWSQEL	HIV	gp160	0.0265
IMIVGGLVGL	HIV	gp160	0.0252
LLYKLDIVSI	HIV	gp160	0.0245
FLAIIWVDL	HIV	gp160	0.0233

Table 26 (Cont'd)

TLQCKIKQII	HIV	gp160	0.0200
GLVGLRIVFA	HIV	gp160	0.0195
FLGAAGSTM	HIV	gp160	0.0190
IISLWDQSL	HIV	gp160	0.0179
TVWGIKQLQA	HIV	gp160	0.0150
LLGRRGWEV	HIV	gp160	0.0142
AVLSIVNRV	HIV	gp160	0.0132
FIMIVGGLV	HIV	gp160	0.0131
LLNATDIAVA	HIV	gp160	0.0117
FLYGALLA	PLP		1.9000
SLLTFMIAA	PLP		0.5300
FMIAATYNFAV	PLP		0.4950
RMYGVLPWI	PLP		0.1650
IAATYNFAV	PLP		0.0540
GLLECCARCLV	PLP		0.0515
YALTVVWLL	PLP		0.0415
ALTVVWLLV	PLP		0.0390
FLYGALLL	PLP		0.0345
SLCADARMYGV	PLP		0.0140
LLVFACSAV	PLP		0.0107

Table 26 (Cont'd)

Sequence	Antigen	A2
KMVELVHFL	MAGE2	0.2200
KVAELVHFL	MAGE3	0.0550
RALAETSYV	MAGE1N	0.0100
LVFGIELMEV	MAGE3	0.1100
FLWGPRALA	MAGE1N	0.0420
ALAETSYVKV	MAGE1	0.0150
LVLGTLEEV	HIV	0.0320
LLWKGEHAVV	HIV	0.0360
IIGAETFYV	HIV	0.0260
LMVTVYGV	HIV	0.4400
LLFNILGGWV	HCV	3.5000
LLALLSCLTV	HCV	0.6100
YLVAYQATV	HCV	0.2500
FLLADARV	HCV	0.2300
ILAGYGAGV	HCV	0.2200
YLLPRGPRL	HCV	0.0730
GLLGCITSL	HCV	0.0610
DLMGYIPLV	HCV	0.0550
LLALLSCLTI	HCV	0.0340
VLAALAAAYCL	HCV	0.0110
LLVPFVQWFV	HBV	1.6000
FLLAQFSA	HBV	0.6600
FLLSLGIHL	HBV	0.5200
ALMPYACI	HBV	0.5000
ILLCLIFLL	HBV	0.3000
LLPIFFCLWV	HBV	0.1000
YLHTLWKAGI	HBV	0.0560

Table 26 (Cont'd)

YLHTLWKAGV	HBV	0.1300
------------	-----	--------



Example 13Identification of immunogenic peptides  
in autoantigens

As noted above, the motifs of the present invention  
5 can also be screened in antigens associated with autoimmune  
diseases. Using the motifs identified above for HLA-A2.1  
allele amino acid sequences from myelin proteolipid (PLP),  
myelin basic protein (MBP), glutamic acid decarboxylase (GAD),  
and human collagen types II and IV were analyzed for the  
10 presence of these motifs. Sequences for the antigens were  
obtained from Trifilieff et al., *C.R. Seances Acad. Sci.*  
300:241 (1985); Eyler et al., *J. Biol. Chem.* 246:5770 (1971);  
Yamashita et al. *Biochim. Biophys. Res. Comm.* 192:1347  
(1993); Su et al., *Nucleic Acids Res.* 17:9473 (1989) and  
15 Pihlajaniemi et al. *Proc. Natl. Acad. Sci. USA* 84:940 (1987).  
The identification of motifs was done using the approach  
described in Example 5 and the algorithms of Examples 6 and 7.  
Table 27 provides the results of the search of these antigens.

Using the quantitative binding assays of Example 4,  
20 the peptides are next tested for the ability to bind MHC  
molecules. The ability of the peptides to suppress  
proliferative responses in autoreactive T cells is carried out  
using standard assays for T cell proliferation. For instance,  
methods as described by Miller et al. *Proc. Natl. Acad. Sci.*  
25 *USA*, 89:421 (1992) are suitable.

For further study, animal models of autoimmune  
disease can be used to demonstrate the efficacy of peptides of  
the invention. For instance, in HLA transgenic mice,  
autoimmune model diseases can be induced by injection of MBP,  
30 PLP or spinal cord homogenate (for MS), collagen (for  
arthritis). In addition, some mice become spontaneously  
affected by autoimmune disease (e.g., NOD mice in diabetes).  
Peptides of the invention are injected into the appropriate  
animals, to identify preferred peptides.

35

TABLE 27  
Human PLP peptides

Pos	AA	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Allele	Motif
3	9	L	L	E	C	C	A	R	C	L		A2.1	(LM)2; (LVI)c
23	9	G	L	C	F	F	G	V	A	L			
39	9	A	L	T	G	T	E	K	L	I			
134	9	S	L	E	R	V	C	H	C	L			
145	9	W	L	G	H	P	D	K	F	V			
158	9	A	L	T	V	V	W	L	L	V			
164	9	L	L	V	F	A	C	S	A	V			
205	9	R	M	Y	G	V	L	P	W	I			
2	10	G	L	L	E	C	C	A	R	C	L		
3	10	L	L	E	C	C	A	R	C	L	V		
10	10	C	L	V	G	A	P	F	A	S	L		
163	10	W	L	L	V	F	A	C	S	A	V		
250	10	T	L	V	S	L	L	T	F	M	I		
64	9	V	I	H	A	F	Q	Y	V	I			Algorithm
80	9	F	L	Y	G	A	L	L	L	A			
157	9	Y	A	L	T	V	V	W	L	L			
163	9	W	L	L	V	F	A	C	S	A			
234	9	Q	M	T	F	H	L	F	I	A			
251	9	L	V	S	L	L	T	F	M	I			
253	9	S	L	L	T	F	M	I	A	A			
259	9	I	A	A	T	Y	N	F	A	V			
84	10	A	L	L	L	A	E	G	F	Y	T		
157	10	Y	A	L	T	V	V	W	L	L	V		
165	10	L	V	F	A	C	S	A	V	P	V		
218	10	K	V	C	G	S	N	L	L	S	I		
253	10	S	L	L	T	F	M	I	A	A	T		

Table 27 continued

## Human Collagen TypeIV peptides

Pos	AA	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Allele	Motif
5	9	A	L	M	G	P	L	G	L	L		A2.1	(LM)2; (LVI)c
11	9	G	L	L	G	Q	I	G	P	L			
23	9	G	M	L	G	Q	K	G	E	I			
231	9	P	L	G	Q	D	G	L	P	V			
3	10	T	L	A	L	M	G	P	L	G	L		
24	10	M	L	G	Q	K	G	E	I	G	L		
59	10	P	L	G	K	D	G	P	P	G	V		
139	10	P	L	G	L	P	G	A	S	G	L		

## Human Collagen TypeII peptides

Pos	AA	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Allele	Motif
794	9	G	L	A	G	Q	R	G	I	V		A2.1	(LM)2; (LVI)c
17	9	V	M	Q	G	P	M	G	P	M			Algorithm

Table 27 continued

Human GAD peptides

Pos	AA	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Allele	Motif
56	9	S	L	E	E	K	S	R	L	V		A2.1	(LM)2; (LVI)c
116	9	F	L	L	E	V	V	D	I	L			
117	9	L	L	E	V	V	D	I	L	L			
150	9	G	M	E	G	F	N	L	E	L			
157	9	E	L	S	D	H	P	E	S	L			
168	9	I	L	V	D	C	R	D	T	L			
190	9	Q	L	S	T	G	L	D	I	I			
229	9	T	L	K	K	M	R	E	I	V			
275	9	G	M	A	A	V	P	K	L	V		A2.1	(LM)2; (LVI)c
300	9	A	L	G	F	G	T	D	N	V			
409	9	V	L	L	F	C	S	A	I	L			
410	9	L	L	Q	C	S	A	I	L	V			
416	9	I	L	V	K	E	K	G	I	L			
466	9	L	M	W	K	A	K	G	T	V			
534	9	K	L	H	K	V	A	P	K	I			
546	9	M	M	E	S	G	T	T	M	V			
582	9	F	L	I	E	E	I	E	R	L			
42	10	K	L	G	L	K	I	C	G	F	L		
116	10	F	L	L	E	V	V	D	I	L	L		
138	10	V	L	D	F	H	H	P	H	Q	L		
147	10	L	L	E	G	M	E	G	F	N	L		
212	10	N	M	F	T	Y	E	I	A	P	V		
275	10	G	M	A	A	V	P	K	L	V	L		
300	10	A	L	G	F	G	T	D	N	V	I		
328	10	I	L	E	A	K	Q	K	G	Y	V		
381	10	L	M	S	R	K	H	R	H	K	L		
409	10	V	L	L	Q	C	S	A	I	L	V		
435	10	L	L	Q	P	D	K	Q	Y	D	V		
465	10	W	L	M	W	K	A	K	G	T	V		
485	10	E	L	A	E	Y	L	Y	A	K	I		
545	10	L	M	M	E	S	G	T	T	M	V		
252	9	G	A	I	S	N	M	Y	S	I			
367	9	N	L	W	L	H	V	D	A	A			
567	9	R	M	V	I	S	N	P	A	A			
299	10	A	A	L	G	F	G	T	D	N	V		
406	10	M	M	G	V	L	L	Q	C	S	A		
423	10	I	L	Q	G	C	N	Q	M	C	A		

Algorithm

## Example 14

Immunogenicity of HPV peptides in A2.1 transgenic mice

5 A group of 14 HPV peptides, including 9 potential epitopes plus 3 low binding and one non-binding peptides as controls was screened for immunogenicity in HLA-A2.1 transgenic mice using the methods described in Example 10. To test the immunogenic potential of the peptides, HLA A2.1 transgenic mice were injected with 50  $\mu$ g/mouse of each HPV peptide together with 140  $\mu$ g/mouse of helper peptide (HBV core 10 128-140 (TPPAYRPPNAPIL). The peptides were injected in the base of the tail in a 1:1 emulsion IFA. Three mice per group were used. As a positive control, the HBV polymerase 561-570 peptide, which induced a strong CTL response in previous experiments, was utilized.

15 Based on these results (Table 28), four unrelated peptides were considered to be the most immunogenic: TLGIVCPI, LLMGTLGIV, YMLDLQPETT, and TIHDIILECV. TLGIVCPI and YMLDLQPETT were found to be good HLA-A2.1 binders, while LLMGTLGIV and TIHDIILECV were found to be intermediate binders 20 in previous binding assays.

TABLE 28

HPV-16 Peptides for possible use in clinical trial

Peptide Position/ Cytel ID	Sequence	AA	A2.1 binding	Immunogenicity Experiment 1	Immunogenicity Experiment 2
E7.86/1088.01	TLGIVCPI	8	0.15	94.4 (1.34)	54.2 (1.43)*
E7.86/1088.06	TLGIVCPIC	9	0.075	2.05 (4.93)	1.3 (3.74)
E7.85/1088.08	GTLGIVCPI	9	0.021	9/08 (3.93)	-**
E7.11/1088.03	YMLDLQPETT	10	0.15	10.32 (1.66)	5.7 (2.39)
E7.11/1088.04	YMLDLQPET	9	0.14	5.0 (3.70)	2.6 (15.5)
E7.12/1088.09	MLDLQPETT	9	0.0028	-	-
E6.52/1088.05	FAFRDLCIV	9	0.057	-	ND
E7.82/1088.02	LLMGTLGIV	9	0.024	9.62 (2.53)	8.93 (1.91)
E6.29/1088.10	TIHDIILECV	10	0.021	22.13 (3.71)	0.4 (3.52)
E7.7/1088.07	TLHEYMLDL	9	0.0070	-	1.2 (3.88)
E6.18/1088.15	KLPQLCTEL	9	0.0009	-	0.3 (5.64)
E6.7/1088.11	AMFQDPQER	10	0.0002	-	ND
E6.26/1088.12	LQTTIHDII	9	0.0002	-	-
E7.73/1088.13	HVDIRTLED	9	0	-	ND

\* Δ Lytic Units, geometric mean x+ SD (3 mice/peptide)

\*\* a dash indicates Δ Lytic Units with a geometric mean ≤0.2

Mixtures of selected HPV epitopes

A combination of CTL peptides and a helper peptide were tested for the ability to provide an increased immune response. The four single peptides were injected separately in order to compare their immunogenicity to injections containing only the two good binders or only the two intermediate binders. In addition all four peptide were injected together. To further evaluate the immunogenicity of a combination of peptides with different binding affinity decreases, another control was introduced in this experiment. A mixture of the two good binders was injected in a different site than the mixture of the two intermediate binders into the base of the tail of the same mouse. All groups of CTL epitopes were injected together with the HBVc helper epitope, with the exception of two groups in which all four HPV coinjected with two different doses of a PADRE helper peptide (aKXVAAWTLKAAa, where a is d-alanine and X is cyclohexylalanine) either 1 $\mu$ g or 0.05 $\mu$ g per mouse.

All four peptides induced a strong CTL response when injected alone and tested using target cells labeled with the appropriate peptide (Table 29). TLGIVCPI proved to be the strongest epitope, an observation confirming the results described above. When mixtures of all four peptides were injected and the responses were stimulated in vitro and tested with target cells pulsed with each single peptide, all combinations showed a strong CTL response. No significant difference was observed when the two helper epitopes were compared. This might in part be due to the fact that the highest dose of PADRE used in this experiment was 140-fold lower than the one for the HBV helper peptide.

Injection of mixtures of the two good binders together or the two intermediate binders resulted in a very low CTL response in both cases even though the single peptides were highly effective. These results, however, are due to a very low number of cell recovery after splenocyte culture of 6 days and are therefore regarded as preliminary.

98  
TABLE 29

## HPV Peptides single and in combinations

A

5

10

15

20

25

Peptide/s injected	Peptides in restimulation and CTL assay			
	1088.01	1088.02	1088.03	1088.10
same as in vitro	116.1 (3.49) *	55.98 (2.49)	5.56 (1.75)	16.4 (1.49)
1088.01 + 1088.03 + 875.23	1.37 (16.56)		0 (0)	
1088.02 + 1088.10 + 875.23		1.11 (2.9)		1.62 (13.1)
1088.01/.03 + 1088.02/.10 + 875.23	19.5 (4.1)	4.68 (2.3)	1.13 (21.9)	1.17 (2.58)
1088.all + 875.23	107.9 (4.77)	13.52 (1.4)	2.58 (5.07)	102.3 (1.32)
1088.all + PADRE 1 µg	73.11 (4.48)	16.83 (2.54)	3.55 (2.9)	20.13 (1.05)
1088.all + PADRE 0.05 µg	37.15 (2.25)	26.79 (2.09)	6.5 (1.64)	4.45 (4.14)

\* Δ Lytic Units 30% geometric mean (+x deviation)

30

35

40

Peptides were dissolved in 50%DMSO/H<sub>2</sub>O to reach a stock concentration of 20mg/ml and were further dissolved in sterile PBS. For subcutaneous injection in the base of the tail of A2.1 transgenic mice, the peptide solution was mixed 1:1 with IFA. The injected amount of HPV-CTL peptides was 50 µg/mouse coinjected with 140 µg/mouse of the HBVcore peptide 875.23 or the indicated dose of PADRE (3 mice/group). Spleens were removed on day 11 and splenocytes were restimulated in vitro with irradiated LPS-Blasts pulsed with the indicated HPV-CTL epitopes at 1µg/ml. After six days, the cytotoxic assay was performed using Jurkat JA2Kb cells (A) or MBB17 (B) as target cells labelled with 51Cr in the presence or absence of the appropriate HPV epitope peptides.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All  
5 publications, patents, and patent applications cited herein are hereby incorporated by reference.



## APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos	A2.1
1.0841	ILSPFLPLL	9	HBV	adr	ENV	371	2.9
1.0240	TLQDIVLHL	9	HPV	18	E7	7	0.76
1.0838	WLSLLVPFV	9	HBV	adr	ENV	335	0.72
1.0851	FLLSLGIHL	9	HBV	adr	POL	1147	0.52
1.0306	QLFEDNYAL	9	c-ERB2			106	0.46
1.0814	LMVTVYYGV	9	HIV		ENV	2182	0.44
1.0878	MMWFWGPSL	9	HBV	adw	ENV	360	0.41
1.0839	MMWYWGPSL	9	HBV	adr	ENV	360	0.41
1.0384	FLTKQYLNL	9	HBV	adw	POL	1279	0.29
1.0321	ILHNGAYSL	9	c-ERB2			435	0.21
1.0834	LLLCLIFLL	9	HBV	adr	ENV	250	0.19
1.0167	GLYSSTVPV	9	HBV	adr	POL	635	0.15
1.0849	HLYSHPIIL	9	HBV	adr	POL	1076	0.13
1.0275	RMPEAAPPV	9	p53			65	0.12
1.0854	LLMGTLGIV	9	HPV	16	E7	82	0.11
1.0880	ILSPFMPLL	9	HBV	adw	ENV	371	0.11
1.0127	YLVAYQATV	9	HCV		LORF	1585	0.11
1.0151	VLLDYQGML	9	HBV	adr	ENV	259	0.11
1.0018	VLAEAMSQV	9	HIV		GAG	367	0.11
1.0330	RLQETELV	9	c-ERB2			689	0.091
1.0209	SLYAVSPSV	9	HBV	adr	POL	1388	0.078
1.0816	DLMGYIPLV	9	HCV		CORE	132	0.055
1.0835	LLCLIFLLV	9	HBV	adr	ENV	251	0.049
1.0852	FLCQQYLHL	9	HBV	adr	POL	1250	0.048

## APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0882	NLYVSLMLL	9	HBV	adw	POL	1088	0.046
1.0837	GMLPVCPLL	9	HBV	adr	ENV	265	0.046
1.0819	ILPCSFTTL	9	HCV		NS1/ENV2	676	0.045
1.0109	ALSTGLIHL	9	HCV		NS1/ENV2	686	0.042
1.0833	ILLCLIFL	9	HBV	adr	ENV	249	0.035
1.0301	HLYQGCQVV	9	c-ERB2			48	0.034
1.0337	CLTSTVQLV	9	c-ERB2			789	0.034
1.0842	PLLPIFFCL	9	HBV	adr	ENV	377	0.031
1.0861	ALCRWGLLL	9	c-ERB2			5	0.031
1.0309	VLIQRNPQL	9	c-ERB2			153	0.029
1.0828	VLQAGFFLL	9	HBV	adr	ENV	177	0.024
1.0844	LLWFHISCL	9	HBV	adr	CORE	490	0.024
1.0135	ILAGYGAGV	9	HCV		LORF	1851	0.024
1.0870	QLMPYGCLL	9	c-ERB2			799	0.023
1.0075	LLWKGEHAV	9	HIV		POL	1496	0.023
1.0873	FLGGTPVCL	9	HBV	adw	ENV	204	0.021
1.0323	ALIHNTL	9	c-ERB2			466	0.021
1.0859	VLVHPQWVL	9	PSA			49	0.020
1.0267	KLQCVDLHV	9	PSA			166	0.019
1.0820	VLPCSFTTL	9	HCV		NS1/ENV2	676	0.017
1.0111	HLHQIVDV	9	HCV		NS1/ENV2	693	0.016
1.0103	SMVGNWAKV	9	HCV		ENV1	364	0.016
1.0283	LLGRNSFEV	9	p53			264	0.014
1.0207	GLYRPLLSL	9	HBV	adr	POL	1370	0.014

## APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos	A2.1
1.0389	GLYRPLLRL	9	HBV	adw	POL	1399	0.014
1.0185	NLSWLSLDV	9	HBV	adr	POL	996	0.013
1.0113	FLLADARV	9	HCV		NS1/ENV2	725	0.013
1.0119	YLVTRHADV	9	HCV		LORF	1131	0.011
1.0846	CLTHIVNLL	9	HBV	adr	POL	912	0.010
1.0156	ELMNLATWV	9	HBV	adr	CORE	454	0.010
1.0236	KLPDLCTEL	9	HPV	18	E6	13	0.010
1.0056	ALQDSGLEV	9	HIV		POL	1180	0.0083
1.0375	LLSSDLSWL	9	HBV	adw	POL	1021	0.0081
1.0094	ALAHGVRVL	9	HCV		CORE	150	0.0072
1.0129	TLHGPTPLL	9	HCV		LORF	1617	0.0070
1.0041	KLLRGTKAL	9	HIV		POL	976	0.0069
1.0131	CMSADLEV	9	HCV		LORF	1648	0.0067
1.0872	GLLGPLLVL	9	HBV	adw	ENV	170	0.0066
1.0228	TLHEYMLDL	9	HPV	16	E7	7	0.0059
1.0274	KLLPENNV	9	p53			24	0.0058
1.0043	ILKEPVHGV	9	HIV		POL	1004	0.0055
1.0206	RLGLYRPLL	9	HBV	adr	POL	1368	0.0050
1.0188	GLPRYVARL	9	HBV	adr	POL	1027	0.0050
1.0202	KLIGTDNSV	9	HBV	adr	POL	1317	0.0050
1.0818	FLLALLSCL	9	HCV		CORE	177	0.0046
1.0184	LLSSNLSWL	9	HBV	adr	POL	992	0.0046
1.0102	QLLRIPQAV	9	HCV		ENV1	337	0.0039
1.0114	GLRDLAVAV	9	HCV		LORF	963	0.0034

## APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos	A2.1
1.0005	TLNAWVKVI	9	HIV		GAG	156	0.0032
1.0183	NLQSLTNLL	9	HBV	adr	POL	985	0.0025
1.0359	QLGRKPTPL	9	HBV	adw	ENV	89	0.0025
1.0150	SLDSWWTSL	9	HBV	adr	ENV	194	0.0023
1.0362	ILSKTGDPV	9	HBV	adw	ENV	153	0.0021
1.0866	ILLVVVLGV	9	c-ERB2			661	0.0020
1.0214	LLHKRTLGL	9	HBV	adr	"X"	1510	0.0019
1.0216	CLFKDWEEL	9	HBV	adr	"X"	1533	0.0019
1.0862	GLGISWLGL	9	c-ERB2			447	0.0018
1.0187	HLLVGSSGL	9	HBV	adr	POL	1020	0.0018
1.0318	TLEEITGYL	9	c-ERB2			402	0.0018
1.0328	PLTSIISAV	9	c-ERB2			650	0.0015
1.0822	LLGCIITSL	9	HCV		LORF	1039	0.0015
1.0277	ALNKMFCQL	9	p53			129	0.0013
1.0066	HLEGKIILV	9	HIV		POL	1322	0.0010
1.0308	QLRSLTEIL	9	c-ERB2			141	0.0008
1.0115	DLAVAVEPV	9	HCV		LORF	966	0.0008
1.0391	VLHKRTLGL	9	HBV	adw	"X"	1539	0.0007
1.0876	FLCILLLCL	9	HBV	adw	ENV	246	0.0007
1.0148	LLDPRVRGL	9	HBV	adr	ENV	120	0.0006
1.0221	KLPQLCTEL	9	HPV	16	E6	18	0.0006
1.0065	HLEGKVILV	9	HIV		POL	1322	0.0006
1.0017	EMMTACQGV	9	HIV		GAG	350	0.0006
1.0055	HLALQDSGL	9	HIV		POL	1178	0.0005

## APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pop.	A2. 1
1.0868	VLGVVFGIL	9	c-ERB2			666	0.0005
1.0004	TLNAWVKVV	9	HIV		GAG	156	0.0005
1.0381	HLESLYAAV	9	HBV	adw	POL	1165	0.0005
1.0128	CLIRLKPTL	9	HCV		LORF	1610	0.0004
1.0255	CLGLSYDGL	9	MAGE	1/3		174	0.0004
1.0212	HLSLRGLPV	9	HBV	adr	"X"	1470	0.0004
1.0247	ILESIFRAV	9	MAGE	1		93	0.0004
1.0092	TLTCGFADL	9	HCV		CORE	125	0.0003
1.0108	TLPALSTGL	9	HCV		NS1/ENV2	683	0.0003
1.0294	ALAIPQCRL	9	EBNA1			525	0.0003
1.0101	DLCGSVFLV	9	HCV		ENV1	280	0.0003
1.0231	RLCVQSTHV	9	HPV	16	E7	66	0.0003
1.0162	LLDDEAGPL	9	HBV	adr	POL	587	0.0002
1.0829	CLRRFIIFL	9	HBV	adr	ENV	239	0.0002
1.0126	GLPVCQDHL	9	HCV		LORF	1547	0.0001
1.0163	PLEEELPRL	9	HBV	adr	POL	594	0.0001
1.0130	PLLYRLGAV	9	HCV		LORF	1623	0.0001
1.0042	ELAENREIL	9	HIV		POL	997	0
1.0054	ELQAIHLAL	9	HIV		POL	1173	0
1.0089	LIPRRGPRL	9	HCV		CORE	36	0
1.0091	NLGKVIDTL	9	HCV		CORE	118	0
1.0093	PLGGAARAL	9	HCV		CORE	143	0
1.0154	DLLDTASAL	9	HBV	adr	CORE	419	0
1.0178	QLKQSRLGL	9	HBV	adr	POL	791	0

## APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2-1
1.0179	GLQPQQGSL	9	HBV	adr	POL	798	0
1.0286	PLDGEYFTL	9	p53			322	0
1.0296	VLKDAIKDL	9	EBNA1			574	0
1.0310	QLCYQDTIL	9	c-ERB2			160	0
1.0007	DLNTMLNTV	9	HIV		GAG	188	0
1.0037	ELHPDKWTV	9	HIV		POL	928	0
1.0070	ELKKIIGQV	9	HIV		POL	1412	0
1.0157	ELVVSIVNV	9	HBV	adr	CORE	473	0
1.0160	CLTFGRETV	9	HBV	adr	CORE	497	0
1.0164	DLNLGNLNV	9	HBV	adr	POL	614	0
1.0867	LLVVVLGVV	9	c-ERB2			662	0
1.0159	NMGLKIRQL	9	HBV	adr	CORE	482	0
1.0322	SLRELGSGL	9	c-ERB2			457	<0.0002
1.0350	DLLEKGERL	9	c-ERB2			933	<0.0002
1.0352	DLVDAEEYL	9	c-ERB2			1016	<0.0002
1.0366	PLEEELPHL	9	HBV	adw	POL	623	<0.0002
1.0372	DLQHGRVLV	9	HBV	adw	POL	781	<0.0002
1.0390	PLPGPLGAL	9	HBV	adw	"X"	1476	<0.0002
1.0811	LLTQIGCTL	9	HIV		POL	685	<0.0002
1.0812	PLVKLWYQL	9	HIV		POL	1116	<0.0002
1.0832	FLFILLLCL	9	HBV	adr	ENV	246	<0.0002
1.0847	NLYVSLLLL	9	HBV	adr	POL	1059	<0.0002
1.0316	PLQPEQLQV	9	c-ERB2			391	<0.0002
1.0342	DLAARNVLV	9	c-ERB2			845	<0.0002

## APPENDIX I: 9-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0343	VLVKSPNHV	9	c-ERB2			851	<0.0002
1.0356	TLSPGKNGV	9	c-ERB2			1172	<0.0002
1.0376	DLSWLSLDV	9	HBV	adw	POL	1025	<0.0002
1.0363	NMENIASGL	9	HBV	adw	ENV	163	<0.0002
1.0195	TLPQEHIVL	9	HBV	adr	POL	1179	<0.0003
1.0196	KLKQCFRKL	9	HBV	adr	POL	1188	<0.0003
1.0201	PLPIHTAEL	9	HBV	adr	POL	1296	<0.0003
1.0210	QLDPARDVL	9	HBV	adr	"X"	1426	<0.0003
1.0220	VLGGCRHKL	9	HBV	adr	"X"	1551	<0.0003
1.0229	DLQPETTDL	9	HPV	16	E7	14	<0.0003
1.0245	ALEAQQEAL	9	MAGE	1		15	<0.0003
1.0266	DLPTQEPAL	9	PSA			136	<0.0003
1.0279	HLIRVEGNL	9	p53			193	<0.0003
1.0282	TLEDSSGNL	9	p53			256	<0.0003
1.0238	ELRHYSDSV	9	HPV	18	E6	77	<0.0003
1.0268	DLHVISNDV	9	PSA			171	<0.0003
1.0836	CLIFLLVLL	9	HBV	adr	ENV	253	<0.0006

## APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0890	LLFNILGGWV	10	HCV		LORF	1807	3.5
1.0930	LLVPFVQWFV	10	HBV	adw	ENV	338	1.6
1.0884	LLALLSCLTV	10	HCV		CORE	178	0.61
1.0896	ILLCLIFLL	10	HBV	adr	ENV	249	0.30
1.0518	GLSPTVWLSV	10	HBV	adr	ENV	348	0.28
1.0902	SLYNILSPFL	10	HBV	adr	ENV	367	0.23
1.0892	LLVLQAGFFL	10	HBV	adr	ENV	175	0.21
1.0686	FLQTHIFAEV	10	EBNA1			565	0.17
1.0628	QLFLNTLSFV	10	HPV	18	E7	88	0.11
1.0904	LLPIFFCLWV	10	HBV	adr	ENV	378	0.10
1.0897	LLLCLIFLLV	10	HBV	adr	ENV	250	0.099
1.0516	LLDYQGMLPV	10	HBV	adr	ENV	260	0.085
1.0901	WMMWYWGPSL	10	HBV	adr	ENV	359	0.084
1.0533	GLYSSTVPVL	10	HBV	adr	POL	635	0.080
1.0469	YLLPRRGPRL	10	HCV		CORE	35	0.073
1.0888	GLLGCITSL	10	HCV		LORF	1038	0.061
1.0907	ILCWGELMNL	10	HBV	adr	CORE	449	0.052
1.0927	LLGICLTSTV	10	c-ERB2			785	0.049
1.0452	LLWKGEHAVV	10	HIV		POL	1496	0.036
1.0885	LLALLSCLTI	10	HCV		CORE	178	0.034
1.0620	KLTNTGLYNL	10	HPV	18	E6	92	0.032
1.0502	RLIVFPDLGV	10	HCV		LORF	2578	0.032
1.0659	FLTPKKLQCV	10	PSA			161	0.031
1.0932	WMMWFWGPSL	10	HBV	adw	ENV	359	0.029



## APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0772	SLNFLGGTPV	10	HBV	adw	ENV	201	0.027
1.0609	SLQDIBITCV	10	HPV	18	E6	24	0.025
1.0526	ILSTLPETTV	10	HBV	adr	CORE	529	0.022
1.0508	RLHGLSAFSL	10	HCV		LORF	2885	0.020
1.0493	ILGGWVAAQL	10	HCV		LORF	1811	0.018
1.0738	VMAGVGSPYV	10	c-ERB2			773	0.018
1.0460	QLMVTVYYGV	10	HIV		ENV	2181	0.017
1.0573	ILRGTSFVYV	10	HBV	adr	POL	1345	0.016
1.0703	SLTEILKGGV	10	c-ERB2			144	0.015
1.0912	LLGCAANWIL	10	HBV	adr	POL	1337	0.014
1.0798	ALPPASPSAV	10	HBV	adw	"X"	1483	0.013
1.0908	QLLWFHISCL	10	HBV	adr	CORE	489	0.013
1.0677	NLLGRNSFEV	10	p53			263	0.013
1.0889	VLAALAAYCL	10	HCV		LORF	1666	0.011
1.0528	LLLDDEAGPL	10	HBV	adr	POL	586	0.011
1.0500	IMAKNEVFCV	10	HCV		LORF	2558	0.0088
1.0492	VLVGGVLAAL	10	HCV		LORF	1661	0.0084
1.0898	LLCLIFLLVL	10	HBV	adr	ENV	251	0.0075
1.0458	KLMVTVYYGV	10	HIV		ENV	2181	0.0069
1.0459	NLMVTVYYGV	10	HIV		ENV	2181	0.0067
1.0530	GLSPTVWLSA	10	HBV	adw	ENV	348	0.0067
1.0759	SLPTHDPSP	10	c-ERB2			1100	0.0059
1.0419	VLPEKDSWTV	10	HIV		POL	940	0.0056
1.0666	FLHSGTAKSV	10	p53			113	0.0050

## APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2-1
1.0473	GLIHLHQIV	10	HCV		NS1/ENV2	690	0.0047
1.0792	SLYAAVTNFL	10	HBV	adw	POL	1168	0.0046
1.0780	IMPARFYPNV	10	HBV	adw	POL	713	0.0043
1.0507	YLTRDPTTPL	10	HCV		LORF	2803	0.0042
1.0914	GLYNLLIRCL	10	HPV	18	E6	97	0.0036
1.0649	YLEYGRCRTV	10	MAGE	1		248	0.0034
1.0561	SLFTSITNFL	10	HBV	adr	POL	1139	0.0034
1.0788	NLLSSDLSWL	10	HBV	adw	POL	1020	0.0032
1.0753	RMARDPQRFV	10	c-ERB2			978	0.0020
1.0568	RMRGTFVVPL	10	HBV	adr	POL	1288	0.0020
1.0642	SLQLVFGIDV	10	MAGE	1		150	0.0020
1.0582	KLLHKRTLGL	10	HBV	adr	'X'	1509	0.0019
1.0713	GLGMEHLREV	10	c-ERB2			344	0.0017
1.0742	GMSYLEDVRL	10	c-ERB2			832	0.0017
1.0549	NLLSSNLSWL	10	HBV	adr	POL	991	0.0016
1.0465	QLTVWGIKQL	10	HIV		ENV	2760	0.0015
1.0524	VLEYLVSGV	10	HBV	adr	CORE	505	0.0015
1.0483	VLNPSVAATL	10	HCV		LORF	1253	0.0015
1.0548	SLTNLLSSNL	10	HBV	adr	POL	988	0.0014
1.0512	ALLDPRVRGL	10	HBV	adr	ENV	119	0.0011
1.0676	TLEDSSGNLL	10	p53			256	0.0011
1.0719	TLQGLGISWL	10	c-ERB2			444	0.0011
1.0627	DLRAFQQLFL	10	HPV	18	E7	82	0.0010
1.0725	VLQGLPREYV	10	c-ERB2			546	0.0009

## APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0918	DLPPWFPPMV	10	EBNA1			605	0.0009
1.0499	DLSDGSWSTV	10	HCV		LORF	2399	0.0008
1.0559	CLAFSYMDDV	10	HBV	adr	POL	1118	0.0008
1.0632	PLVLGTLEEV	10	MAGE	1		37	0.0008
1.0520	NLATWVGSNL	10	HBV	adr	CORE	457	0.0008
1.0400	NLLTQIGCTL	10	HIV		POL	684	0.0007
1.0488	GLTHIDAHFL	10	HCV		LORF	1564	0.0007
1.0733	VLGSGAFGTV	10	c-ERB2			725	0.0007
1.0434	QLIKKEKVYL	10	HIV		POL	1219	0.0006
1.0451	KLLWKGEHAV	10	HIV		POL	1495	0.0006
1.0470	SMVGNWAKVL	10	HCV		ENV1	364	0.0006
1.0570	KLIGTDNSVV	10	HBV	adr	POL	1317	0.0006
1.0924	ILLVVVLGVV	10	c-ERB2			661	0.0006
1.0397	LLDTGADDTV	10	HIV		POL	619	0.0005
1.0446	HLKTAVQMAV	10	HIV		POL	1426	0.0005
1.0604	DLLMGTLGIV	10	HPV	16	E7	81	0.0005
1.0443	LLKLAGRWPV	10	HIV		POL	1356	0.0004
1.0461	DLMVTVYYGV	10	HIV		ENV	2181	0.0004
1.0619	TLEKLTNTGL	10	HPV	18	E6	89	0.0004
1.0787	SLTNLLSSDL	10	HBV	adw	POL	1017	0.0004
1.0521	NLEDPASREL	10	HBV	adr	CORE	465	0.0003
1.0583	GLSAMSTTDL	10	HBV	adr	'X'	1517	0.0003
1.0652	VLVASRGRAV	10	PSA			36	0.0003
1.0716	DLSVFQNLQV	10	c-ERB2			421	0.0003

## APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2.1
1.0723	QLFRNPHQAL	10	c-ERB2			484	0.0003
1.0727	PLTSIISAVV	10	c-ERB2			650	0.0003
1.0479	YLKGSSGGPL	10	HCV		LORF	1160	0.0002
1.0497	QLPCEPEPDV	10	HCV		LORF	2159	0.0002
1.0523	CLTFGRETVL	10	HBV	adr	CORE	497	0.0002
1.0603	TLEDLLMGTL	10	HPV	16	E7	78	0.0002
1.0631	SLHCKPEEAL	10	MAGE	1		7	0.0002
1.0680	EMFRELNEAL	10	p53			339	0.0002
1.0689	VLKDAIKDLV	10	EBNA1			574	0.0002
1.0757	DLVDAEEYLV	10	c-ERB2			1016	0.0002
1.0796	RMRGTFVSPL	10	HBV	adw	POL	1317	0.0002
1.0669	QLAKTCPVQL	10	p53			136	0.0001
1.0717	NLQVIRGRIL	10	c-ERB2			427	0.0001
1.0721	WLGLRSLREL	10	c-ERB2			452	0.0001
1.0522	NMGLKIRQLL	10	HBV	adr	CORE	482	0
1.0527	PLSYQHFRKL	10	HBV	adr	POL	576	0
1.0529	ELPRLADEGL	10	HBV	adr	POL	598	0
1.0531	GLNRRVAEDL	10	HBV	adr	POL	606	0
1.0536	PLTVNEKRRL	10	HBV	adr	POL	672	0
1.0539	IMPARFYPNL	10	HBV	adr	POL	684	0
1.0550	PLHPAAMPHL	10	HBV	adr	POL	1012	0
1.0552	DLHDSCSRNL	10	HBV	adr	POL	1051	0
1.0555	LLYKTFRKRL	10	HBV	adr	POL	1066	0
1.0557	PMGVGLSPFL	10	HBV	adr	POL	1090	0

## APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos	A2.1
1.0560	VLGAKSVQHL	10	HBV	adr	POL	1128	0
1.0569	PLPIHTAELL	10	HBV	adr	POL	1296	0
1.0579	PLPSLAFSAV	10	HBV	adr	'X'	1454	0
1.0585	DLEAYFKDCL	10	HBV	adr	'X'	1525	0
1.0587	ELGEEIRLKV	10	HBV	adr	'X'	1540	0
1.0589	VLGGCRHKL	10	HBV	adr	'X'	1551	0
1.0597	TLEQQYNKPL	10	HPV	16	E6	94	0
1.0608	DLCTELNTSL	10	HPV	18	E6	16	0
1.0616	RLQRRRETQV	10	HPV	18	E6	49	0
1.0621	HLEPQNEIPV	10	HPV	18	E7	14	0
1.0639	LLKYRAREPV	10	MAGE	1/3		114	0
1.0643	CLGLSYDGLL	10	MAGE	1/3		174	0
1.0657	DMSLLKNRFL	10	PSA			98	0
1.0658	LLRLSEPAEL	10	PSA			119	0
1.0663	PLSQETFSDL	10	p53			13	0
1.0664	PLPSQAMDDL	10	p53			34	0
1.0690	ELAALCRWGL	10	c-ERB2			2	0
1.0692	RLPASPETHL	10	c-ERB2			34	0
1.0699	RLRIVRGTQL	10	c-ERB2			98	0
1.0701	GLRELQLRSL	10	c-ERB2			136	0
1.0730	QMRILKETEL	10	c-ERB2			711	0
1.0732	ILKETELRKV	10	c-ERB2			714	0
1.0754	PLDSTFYRSL	10	c-ERB2			999	0
1.0755	LLEDDDMGDL	10	c-ERB2			1008	0

## APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos	A2.1
1.0758	DLGMGAAKGL	10	c-ERB2			1089	9
1.0761	PLPSETDGYV	10	c-ERB2			1119	0
1.0763	TLSPGKNGVV	10	c-ERB2			1172	0
1.0765	TLQDPRVRAL	10	HBV	adw	ENV	119	0
1.0768	NMENIASGLL	10	HBV	adw	ENV	163	0
1.0775	ELPHLADEGL	10	HBV	adw	POL	627	0
1.0776	GLNRPVAEDL	10	HBV	adw	POL	635	0
1.0777	PLTVNENRRL	10	HBV	adw	POL	701	0
1.0790	LLYKTYGRKL	10	HBV	adw	POL	1095	0
1.0801	GLSAMSP TDL	10	HBV	adw	"X"	1546	0
1.0802	DLEAYFKDCV	10	HBV	adw	"X"	1554	0
1.0803	TLQDPRVRGL	10	HBV	ayw	ENV	119	0
1.0804	NMENITSGFL	10	HBV	ayw	ENV	163	0
1.0891	DLVNLLPAIL	10	HCV		LORF	1878	0
1.0404	PLTEEKIKAL	10	HIV		POL	720	<0.0002
1.0409	QLGIPHPAGL	10	HIV		POL	786	<0.0002
1.0411	GLKKKKS VTV	10	HIV		POL	794	<0.0002
1.0450	PIWKGP AKLL	10	HIV		POL	1488	<0.0002
1.0476	DLAVAVEPVV	10	HCV		LORF	966	<0.0002
1.0478	SLTGRDKNQV	10	HCV		LORF	1046	<0.0002
1.0490	DLEVVTSTWV	10	HCV		LORF	1652	<0.0002
1.0494	GLGKVLIDIL	10	HCV		LORF	1843	<0.0002
1.0505	VLT TSCGNTL	10	HCV		LORF	2704	<0.0002
1.0506	ELITSCSSNV	10	HCV		LORF	2781	<0.0002

## APPENDIX II: 10-MER PEPTIDES

Peptide	Sequence	AA	Virus	Strain	Molecule	Pos.	A2-1
1.0510	CLRKLGVPPL	10	HCV		LORF	2908	<0.0002
1.0511	PLGFFPDHQL	10	HBV	adr	ENV	10	<0.0002
1.0514	NMENTTSGFL	10	HBV	adr	ENV	163	<0.0002

Appendix III PLP 8-mers											
Source	Peptide	AA	1	2	3	4	5	6	7	8	Algorithm Score (E02)
Hu PLP	10	8	G	L	V	G	A	P	F	A	
Hu PLP	13	8	G	A	P	F	A	S	L	V	
Hu PLP	23	8	G	L	C	F	F	G	V	A	
Hu PLP	39	8	A	L	T	G	T	E	K	L	
Hu PLP	40	8	L	T	G	T	E	K	L	I	
Hu PLP	60	8	Y	L	I	N	V	I	H	A	
Ms PLP	64	8	V	I	H	A	F	Q	C	V	
Hu PLP	64	8	V	I	H	A	F	Q	Y	V	
Hu PLP	74	8	G	T	A	S	F	F	F	L	
Hu PLP	80	8	F	L	Y	G	A	L	L	L	
Hu PLP	93	8	T	T	G	A	V	R	Q	I	
Hu PLP	106	8	T	T	I	C	G	K	G	L	
Hu PLP	131	8	Q	A	H	S	L	E	R	V	
Hu PLP	152	8	F	V	G	I	T	Y	A	L	
Hu PLP	154	8	G	T	T	Y	A	L	T	V	
Hu PLP	155	8	I	T	Y	A	L	T	V	V	
Hu PLP	157	8	Y	A	L	T	V	V	W	L	
Hu PLP	158	8	A	L	T	V	V	W	L	L	
Hu PLP	159	8	L	T	V	V	W	L	L	V	
Hu PLP	164	8	L	L	V	F	A	C	S	A	
Hu PLP	165	8	L	V	F	A	C	S	A	V	
Hu PLP	152	8	F	A	C	S	A	V	F	V	
Hu PLP	199	8	S	L	C	A	D	A	R	M	
Hu PLP	203	8	D	A	R	M	Y	G	V	L	
Hu PLP	212	8	W	I	A	F	P	G	K	V	
Hu PLP	218	8	K	V	C	G	S	N	L	L	
Hu PLP	224	8	L	L	S	I	C	K	T	A	
Hu PLP	234	8	Q	M	T	F	H	L	F	I	
Hu PLP	238	8	H	L	F	I	A	A	F	V	
Hu PLP	244	8	F	V	G	A	A	A	T	L	
Hu PLP	247	8	A	A	A	T	L	V	S	L	



Appendix III PLP 8-mers											
Source	Peptide	AA	1	2	3	4	5	6	7	8	Algorithm Score (E02)
Hu PLP	248	8	A	A	T	L	V	S	L	L	
Hu PLP	253	8	S	L	L	T	F	M	I	A	
Hu PLP	254	8	L	L	T	F	M	I	A	A	
Hu PLP	260	8	A	A	T	Y	N	F	A	V	
Hu PLP	261	8	A	T	Y	N	F	A	V	L	

Appendix III MBP 8-mers											
Source	Peptide	AA	1	2	3	4	5	6	7	8	Algorithm Score (E02)
Hu MBP	14	8	Y	L	A	T	A	S	T	M	
Hu MBP	34	8	D	T	G	I	L	D	S	I	
Hu MBP	65	8	R	T	A	H	Y	G	S	L	
Ms MBP	70	8	H	A	R	S	R	P	G	L	
Hu MBP	79	8	R	T	Q	D	E	N	P	V	
Hu MBP	86	8	V	V	H	F	F	K	N	I	
Ms MBP	87	8	R	T	T	H	Y	G	S	L	
Hu MBP	143	8	G	V	D	A	Q	G	T	L	
Hu MBP	149	8	T	L	S	K	I	F	K	L	

Appendix III PLP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (E02)
Hu PLP	163	9	W	L	L	V	F	A	C	S	A	-18.67
Hu PLP	205	9	R	M	Y	G	V	L	P	W	I	-18.79
Hu PLP	145	9	W	L	G	H	P	D	K	F	V	-19.05
Hu PLP	253	9	S	L	L	T	F	M	I	A	A	-19.07
Hu PLP	251	9	L	V	S	L	L	T	F	M	I	-20.03
Hu PLP	258	9	M	I	A	A	T	Y	N	P	A	-20.32
Hu PLP	80	9	F	L	Y	G	A	L	L	L	A	-20.53
Ms PLP	205	9	F	M	Y	G	V	L	P	W	N	-20.69
Hu PLP	64	9	V	I	H	A	F	Q	Y	V	I	-20.71
Hu PLP	23	9	G	L	C	F	F	G	V	A	L	-21.23
Ms PLP	23	9	G	L	C	F	F	G	V	A	L	-21.23
Ms PLP	179	9	N	T	W	T	T	C	Q	S	I	-21.24
Hu PLP	233	9	F	Q	M	T	F	H	L	F	I	-21.25
Hu PLP	234	9	Q	M	T	F	H	L	F	I	A	-21.29
Hu PLP	250	9	I	A	A	T	Y	N	F	A	V	-21.32
Hu PLP	157	9	Y	A	L	T	V	V	W	L	L	-21.51
Hu PLP	76	9	A	S	F	F	F	L	Y	G	A	-21.52
Hu PLP	158	9	A	L	T	V	V	W	L	L	V	-21.56
Hu PLP	252	9	V	S	L	L	T	F	M	I	A	-21.58
Hu PLP	237	9	F	H	L	F	I	A	A	F	V	-21.61
Ms PLP	208	9	G	V	L	P	W	N	A	F	P	-21.61
Hu PLP	164	9	L	L	V	F	A	C	S	A	V	-21.81
Hu PLP	78	9	F	F	F	L	Y	G	A	L	L	-22.05
Hu PLP	250	9	T	L	V	S	L	L	T	F	M	-22.10
Hu PLP	208	9	G	V	L	P	W	I	A	F	P	-22.10
Hu PLP	39	9	A	L	T	G	T	E	K	L	I	-22.13
Hu PLP	240	9	F	I	A	A	F	V	G	A	A	-22.19
Hu PLP	235	9	M	T	F	H	L	F	I	A	A	-22.22
Hu PLP	244	9	F	V	G	A	A	A	T	L	V	-22.22
Ms PLP	64	9	V	I	H	A	F	Q	C	V	I	-22.33

Appendix III PLP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (EO2)
Hu PLP	12	9	V	G	A	P	F	A	S	L	V	-22.36
Hu PLP	45	9	K	L	I	E	T	Y	F	S	K	-22.42
Hu PLP	30	9	A	L	F	C	G	C	G	H	E	-22.46
Hu PLP	9	9	R	C	L	V	G	A	P	F	A	-22.52
Hu PLP	189	9	F	P	S	K	T	S	A	S	I	-22.54
Hu PLP	71	9	V	I	Y	G	T	A	S	F	F	-22.56
Hu PLP	73	9	Y	G	T	A	S	F	F	F	L	-22.63
Hu PLP	11	9	L	V	G	A	F	F	A	S	L	-22.64
Hu PLP	86	9	L	L	A	E	G	F	Y	T	T	-22.65
Ms PLP	63	9	N	V	I	H	A	F	Q	C	V	-22.65
Hu PLP	212	9	W	I	A	F	P	G	K	V	C	-22.67
Hu PLP	223	9	N	L	L	S	I	C	K	T	A	-22.68
Hu PLP	199	9	S	L	G	A	D	A	R	M	Y	-22.71
Hu PLP	179	9	N	T	W	T	T	C	D	S	I	-22.73
Hu PLP	201	9	C	A	D	A	R	M	Y	G	V	-22.74
Hu PLP	112	9	G	L	S	A	T	V	T	G	G	-22.78
Hu PLP	161	9	V	V	W	L	L	V	F	A	C	-22.78
Hu PLP	175	9	Y	I	Y	F	N	T	W	T	T	-22.81

Appendix III PLP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (EO2)
Hu PLP	56	9	Q	D	Y	E	Y	L	I	N	V	-22.84
Hu PLP	241	9	I	A	A	F	V	G	A	A	A	-22.87
Hu PLP	154	9	G	I	T	Y	A	L	T	V	V	-22.89
Hu PLP	257	9	F	M	I	A	A	T	Y	N	F	-22.89
Hu PLP	196	9	S	I	G	S	L	C	A	D	A	-22.90
Hu PLP	18	9	S	L	V	A	T	G	L	C	F	-22.91
Hu PLP	261	9	A	T	Y	N	F	A	V	L	K	-23.40
Hu PLP	171	9	A	V	P	V	Y	I	Y	F	N	-23.05
Hu PLP	70	9	Y	V	I	Y	G	T	A	S	F	-23.11
Hu PLP	28	9	T	G	L	C	F	F	G	V	A	-23.12
Hu PLP	134	9	S	L	E	R	V	C	H	C	L	-23.16
Hu PLP	16	9	F	A	S	L	V	A	T	G	L	-23.20
Hu PLP	74	9	G	T	A	S	F	F	F	L	Y	-23.20
Hu PLP	79	9	F	F	L	Y	G	A	L	L	L	-23.20
Hu PLP	246	9	G	A	A	A	T	L	V	S	L	-23.26
Hu PLP	181	9	W	T	T	C	D	S	I	A	F	-23.27
Hu PLP	28	9	G	V	A	L	F	C	G	C	G	-23.31
Hu PLP	247	9	A	A	A	T	L	V	S	L	L	-23.31
Hu PLP	219	9	V	C	G	S	N	L	L	S	I	-23.33
Hu PLP	160	9	T	V	V	W	L	L	V	F	A	-23.40
Hu PLP	54	9	N	Y	Q	D	Y	E	Y	L	I	-23.43
Hu PLP	107	9	T	I	C	G	K	G	L	S	A	-23.45
Hu PLP	166	9	V	F	A	G	S	A	V	F	V	-23.53
Hu PLP	2	9	G	L	L	E	C	C	A	R	C	-23.57
Hu PLP	167	9	F	A	C	S	A	V	P	V	Y	-23.60
Hu PLP	260	9	A	A	T	Y	N	F	A	V	L	-23.61
Hu PLP	152	9	F	V	G	I	T	Y	A	L	T	-23.63
Hu PLP	187	9	I	A	F	P	S	K	T	S	A	-23.64
Hu PLP	63	9	N	V	I	H	A	F	Q	Y	V	-23.65
Hu PLP	60	9	Y	L	I	N	V	I	H	A	F	-23.66
Hu PLP	85	9	L	L	L	A	E	G	F	Y	T	-23.66

Appendix III PLP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (E02)
Ms PLP	210	9	L	P	W	N	A	F	P	G	K	-23.66
Hu PLP	198	9	G	S	L	C	A	D	A	R	M	-23.67
Hu PLP	20	9	V	A	T	G	L	C	F	F	G	-23.71
Hu PLP	263	9	Y	N	F	A	V	L	K	L	M	-23.71
Ms PLP	209	9	V	L	P	W	N	A	F	P	G	-23.71
Hu PLP	84	9	A	L	L	L	A	E	G	F	Y	-23.73
Hu PLP	206	9	M	Y	G	V	L	P	W	I	A	-23.77
Hu PLP	153	9	V	G	I	T	Y	A	L	T	V	-23.80
Hu PLP	269	9	K	L	M	G	R	G	T	K	F	-23.92
Hu PLP	198	9	V	C	H	C	L	G	K	W	L	-23.99
Hu PLP	3	9	L	L	E	C	C	A	R	C	L	-24.02
Hu PLP	92	9	Y	T	T	G	A	V	R	Q	I	-24.40
Hu PLP	21	9	A	T	G	L	C	F	F	G	V	-24.47
Hu PLP	192	9	K	T	S	A	S	I	G	S	L	-24.74
Hu PLP	38	9	E	A	L	T	G	T	E	K	L	-25.72
Hu PLP	105	9	K	T	T	I	C	G	K	G	L	-26.97

Appendix III MBP 9-mers												
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	Algorithm Score (EO2)
Hu MBP	110	9	S	L	S	R	F	S	W	G	A	-21.42
Hu MBP	14	9	Y	L	A	T	A	S	T	M	D	-22.01
Ms MBP	59	9	W	L	K	Q	S	R	S	F	L	-22.60
Hu MBP	86	9	V	V	H	F	F	K	N	I	V	-22.80
Ms MBP	52	9	R	G	S	G	K	V	P	W	L	-22.87
Hu MBP	16	9	A	T	A	S	T	M	D	H	A	-23.41
Hu MBP	37	9	I	L	D	S	I	G	R	F	F	-23.11
Hu MBP	108	9	G	L	S	L	S	R	F	S	W	-23.34
Hu MBP	93	9	I	V	T	P	R	T	P	P	P	-23.41
Ms MBP	63	9	S	R	S	P	L	F	S	H	A	-23.47
Hu MBP	79	9	R	T	Q	D	E	N	P	V	V	-23.49
Hu MBP	129	9	G	R	A	S	D	Y	K	S	A	-23.53
Hu MBP	21	9	M	D	H	A	R	H	G	F	L	-23.60
Hu MBP	160	9	D	S	R	S	G	S	R	M	A	-23.63
Ms MBP	79	9	P	G	L	C	H	M	Y	K	D	-23.64
Hu MBP	112	9	S	R	F	S	W	G	A	E	G	-23.77
Hu MBP	160	9	R	S	G	S	P	M	A	R	R	-23.77
Hu MBP	159	9	R	D	S	R	S	G	S	P	M	-23.81
Hu MBP	85	9	F	V	V	H	F	F	K	N	I	-23.82
Hu MBP	136	9	S	A	H	K	G	F	K	G	V	-23.90
Hu MBP	149	9	T	L	S	K	I	F	K	L	G	-23.90
Ms MBP	162	9	K	G	F	K	G	A	Y	D	A	-23.92
Hu MBP	64	9	A	R	T	A	H	Y	G	S	L	-23.99
Ms MBP	166	9	G	A	Y	D	A	Q	G	T	L	-24.66
Hu MBP	148	9	G	T	L	S	K	I	F	K	L	-24.78
Hu MBP	145	9	D	A	Q	G	T	L	S	K	I	-25.25

Appendix III PLP 10-mers													
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	Algorithm Score (E02)
Ms PLP	178	10	F	N	T	W	T	T	C	Q	S	I	-24.68
Hu PLP	178	10	F	N	T	W	T	T	C	D	S	I	-25.14
Hu PLP	204	10	A	R	M	Y	G	V	L	P	W	I	-25.48
Hu PLP	163	10	W	L	L	V	F	A	C	S	A	V	-25.66
Hu PLP	218	10	K	V	C	G	S	N	L	L	S	I	-25.89
Hu PLP	250	10	T	L	V	S	L	L	T	F	M	I	-26.00
Hu PLP	19	10	L	V	A	T	G	L	C	F	F	G	-26.25
Hu PLP	78	10	F	F	F	L	Y	G	A	L	L	L	-26.68
Hu PLP	157	10	Y	A	L	T	V	V	W	L	L	V	-26.72
Hu PLP	84	10	A	L	L	L	A	E	G	F	Y	T	-26.77
Hu PLP	233	10	P	Q	M	T	F	H	L	F	I	A	-26.78
Hu PLP	80	10	F	L	Y	G	A	L	L	L	A	E	-26.79
Hu PLP	167	10	F	A	C	S	A	V	P	V	Y	I	-27.28
Hu PLP	165	10	L	V	F	A	C	S	A	V	P	V	-27.32
Hu PLP	4	10	L	E	C	C	A	R	C	L	V	G	-27.36
Hu PLP	253	10	S	L	L	T	F	M	I	A	A	T	-27.42
Hu PLP	135	10	L	E	R	V	C	H	C	L	G	K	-27.48
Hu PLP	176	10	I	Y	F	N	T	W	T	T	C	D	-27.62
Hu PLP	24	10	L	C	F	F	G	V	A	L	F	C	-27.74
Hu PLP	146	10	L	G	H	P	D	K	F	V	G	I	-27.88
Hu PLP	237	10	F	H	L	F	I	A	A	F	V	G	-27.95
Hu PLP	56	10	Q	D	Y	E	Y	L	I	N	V	I	-27.99
Ms PLP	204	10	A	F	M	Y	G	V	L	F	W	N	-28.01
Hu PLP	158	10	A	L	T	V	V	W	L	L	V	R	-28.04
Hu PLP	137	10	R	V	C	H	C	L	G	K	W	L	-28.15
Hu PLP	72	10	I	Y	G	T	A	S	F	F	F	L	-28.16
Hu PLP	63	10	N	V	I	H	A	F	Q	Y	V	I	-28.17
Hu PLP	208	10	G	V	L	P	W	I	A	F	P	G	-28.17
Hu PLP	27	10	F	G	V	A	L	F	C	G	C	G	-28.29
Hu PLP	85	10	L	L	L	A	E	G	F	Y	T	T	-28.32
Ms PLP	62	10	I	N	V	I	H	A	F	Q	C	V	-28.33



Appendix III PLP 10-mers													
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	Algorithm Score (EO2)
Hu PLP	222	10	S	N	L	L	S	I	C	K	T	A	-28.40
Hu PLP	76	10	A	S	F	F	F	L	Y	G	A	L	-28.43
Ms PLP	208	10	G	V	L	P	W	N	A	F	P	G	-28.45
Hu PLP	207	10	Y	G	V	L	P	W	I	A	F	P	-28.46
Hu PLP	79	10	F	F	L	Y	G	A	L	L	L	A	-28.49
Hu PLP	236	10	T	F	H	L	F	I	A	A	F	V	-28.50
Hu PLP	240	10	F	I	A	A	F	V	G	A	A	A	-28.51
Hu PLP	181	10	W	T	T	C	D	S	I	A	F	P	-28.56
Hu PLP	224	10	L	L	S	I	C	K	T	A	E	F	-28.56
Hu PLP	10	10	C	L	V	G	A	P	F	A	S	L	-28.62
Hu PLP	152	10	F	V	G	I	T	Y	A	L	T	V	-28.64
Hu PLP	62	10	I	N	V	I	H	A	F	Q	Y	V	-28.64
Hu PLP	214	10	A	F	P	G	K	V	C	G	S	N	-28.65
Hu PLP	188	10	A	F	P	S	K	T	S	A	S	I	-28.65
Hu PLP	99	10	Q	I	F	G	D	Y	K	T	T	I	-28.69
Hu PLP	18	10	S	L	V	A	T	G	L	C	F	F	-28.73
Hu PLP	3	10	L	L	E	C	C	A	R	C	L	V	-28.75

Appendix III PLP 10-mers													
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	Algorithm Score (EO2)
Hu PLP	17	10	A	S	L	V	A	T	G	L	C	F	-28.76
Hu PLP	144	10	K	W	L	G	H	P	D	K	F	V	-28.78
Ms PLP	181	10	W	T	T	C	Q	S	I	A	F	F	-28.78
Hu PLP	159	10	L	T	V	V	W	L	L	V	F	A	-28.79
Hu PLP	174	10	V	Y	I	Y	F	N	T	W	T	T	-28.80
Hu PLP	248	10	A	A	T	L	V	S	L	L	T	F	-28.84
Hu PLP	23	10	G	L	C	F	F	G	V	A	L	F	-28.87
Hu PLP	205	10	V	L	P	W	I	A	F	P	G	K	-28.87
Hu PLP	29	10	V	A	L	F	C	G	C	G	H	E	-28.90
Hu PLP	261	10	A	T	Y	N	F	A	V	L	K	L	-28.92
Ms PLP	63	10	N	V	I	H	A	F	Q	C	V	I	-28.93
Hu PLP	74	10	G	T	A	S	F	F	F	L	Y	G	-28.93
Hu PLP	259	10	I	A	A	T	Y	N	F	A	V	L	-29.06
Hu PLP	242	10	A	A	F	V	G	A	A	A	T	L	-29.24
Hu PLP	2	10	G	L	L	E	C	C	A	R	C	L	-29.30
Hu PLP	257	10	F	M	I	A	A	T	Y	N	F	A	-29.37
Hu PLP	20	10	V	A	T	G	L	C	F	F	G	V	-29.41
Ms PLP	205	10	R	M	Y	G	V	L	P	W	N	A	-29.43
Hu PLP	155	10	I	T	Y	A	L	T	V	V	W	L	-29.60
Hu PLP	30	10	A	L	F	C	G	C	G	H	E	A	-29.70
Hu PLP	205	10	R	M	Y	G	V	L	P	W	I	A	-29.74
Hu PLP	258	10	M	I	A	A	T	Y	N	F	A	V	-30.06
Hu PLP	234	10	Q	M	T	F	H	L	F	I	A	A	-30.29
Hu PLP	234	10	H	L	F	I	A	A	F	V	G	A	-30.64
Hu PLP	246	10	G	A	A	A	T	L	V	S	L	L	-30.64
Hu PLP	38	10	E	A	L	T	G	T	E	K	L	I	-30.92
Hu PLP	230	10	T	A	E	F	Q	M	T	F	H	L	-31.03
Hu PLP	11	10	L	V	G	A	P	F	A	S	L	V	-31.25
Hu PLP	201	10	C	A	D	A	R	M	Y	G	V	L	-31.73

Appendix III PLP 11-mers														
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	11	Algorithm Score (EO2)
Hu PLP	2	11	G	L	L	E	C	C	A	R	C	L	V	
Hu PLP	10	11	C	L	V	G	A	P	F	A	S	L	V	
Hu PLP	19	11	L	V	A	T	G	L	C	F	F	G	V	
Hu PLP	21	11	A	T	G	L	C	F	F	G	V	A	L	
Hu PLP	30	11	A	L	F	C	G	C	G	H	E	A	L	
Hu PLP	61	11	L	I	N	V	I	H	A	F	Q	Y	V	
Ms PLP	61	11	L	I	N	V	I	H	A	F	Q	C	V	
Hu PLP	71	11	V	I	Y	G	T	A	S	F	F	F	L	
Hu PLP	75	11	T	A	S	F	F	F	L	Y	G	A	L	
Hu PLP	86	11	L	L	A	E	G	F	Y	T	T	G	A	
Hu PLP	87	11	L	A	E	G	F	Y	T	T	G	A	V	
Hu PLP	107	11	T	I	C	G	K	G	L	S	A	T	V	
Hu PLP	145	11	W	L	G	H	P	D	K	F	V	G	I	
Hu PLP	152	11	F	V	G	I	T	Y	A	L	T	V	V	
Hu PLP	154	11	G	I	T	Y	A	L	T	V	V	W	L	
Hu PLP	155	11	I	T	Y	A	L	T	V	V	W	L	L	
Hu PLP	158	11	A	L	T	V	V	W	L	L	V	F	A	
Hu PLP	164	11	L	L	V	F	A	C	S	A	V	P	V	
Hu PLP	187	11	I	A	F	P	S	K	T	S	A	S	I	
Hu PLP	199	11	S	L	C	A	D	A	R	M	Y	G	V	
Hu PLP	203	11	D	A	R	M	Y	G	V	L	P	W	I	
Hu PLP	209	11	V	L	P	W	I	A	F	P	G	K	V	
Ms PLP	209	11	V	L	P	W	N	A	F	P	G	K	V	
Hu PLP	229	11	K	T	A	E	F	Q	M	T	F	H	L	
Hu PLP	235	11	M	T	F	H	L	F	I	A	A	F	V	
Hu PLP	238	11	H	L	F	I	A	A	F	V	G	A	A	
Hu PLP	241	11	I	A	A	F	V	G	A	A	A	T	L	
Hu PLP	242	11	A	A	F	V	G	A	A	A	T	L	V	
Hu PLP	244	11	F	V	G	A	A	A	T	L	V	S	L	
Hu PLP	249	11	A	T	L	V	S	L	L	T	F	M	I	

127

Appendix III PLP 11-mers														
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	11	Algorithm Score (EO2)
Hu PLP	250	11	T	L	V	S	L	L	T	F	M	I	A	
Hu PLP	257	11	F	M	I	A	A	T	Y	N	F	A	V	
Hu PLP	258	11	M	I	A	A	T	Y	N	F	A	V	L	
Hu PLP	260	11	A	A	T	Y	N	F	A	V	L	K	L	

Appendix III MBP 10-mers													
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	Algorithm Score (EO2)
Hu MBP	37	10	I	L	D	S	I	G	R	F	F	G	-27.66
Hu MBP	28	10	F	L	P	R	H	R	D	T	G	I	-27.85
Ms MBP	167	10	A	Y	D	A	Q	G	T	L	S	K	-28.54
Hu MBP	89	10	F	F	K	N	I	V	T	P	R	T	-28.68
Hu MBP	14	10	Y	L	A	T	A	S	T	M	D	H	-28.75
Hu MBP	84	10	N	P	V	V	H	F	F	K	N	I	-28.90
Hu MBP	32	10	H	R	D	T	G	I	L	D	S	I	-28.83
Hu MBP	110	10	S	L	S	R	F	S	W	G	A	E	-28.98
Hu MBP	85	10	P	V	V	H	F	F	K	N	I	V	-30.82
Ms MBP	85	10	H	T	R	T	T	H	Y	G	S	L	-31.29
Hu MBP	20	10	T	M	D	H	A	R	H	G	F	L	-31.40
Hu MBP	63	10	P	A	R	T	A	H	Y	G	S	L	-31.76
Ms MBP	48	10	G	A	P	K	R	G	S	G	K	V	-32.21

Appendix III MBP 11-mers														
Source	Peptide	AA	1	2	3	4	5	6	7	8	9	10	11	Algorithm Score (EO2)
Hu MBP	14	11	Y	L	A	T	A	S	T	M	D	H	A	
Hu MBP	19	11	S	T	M	D	H	A	R	H	G	F	L	
Hu MBP	28	11	F	L	P	R	H	R	D	T	G	I	L	
Hu MBP	108	11	G	L	S	L	S	R	F	S	W	G	A	
Hu MBP	143	11	G	V	D	A	Q	G	T	L	S	K	I	

WHAT IS CLAIMED IS:

1. A composition comprising an immunogenic peptide having an HLA-A2.1 binding motif, which immunogenic peptide  
5 has 9 residues and the following residues:

a first conserved residue at the second position from the N-terminus selected from the group consisting of I, V, A and T;

10 a second conserved residue at the C-terminal position selected from the group consisting of V, L, I, A and M.

2. A composition comprising an immunogenic peptide having an HLA-A2.1 binding motif, which immunogenic peptide  
15 has 9 residues:

a first conserved residue at the second position from the N-terminus selected from the group consisting of L, M, I, V, A and T;

20 a second conserved residue at the C-terminal position selected from the group consisting of A and M;

3. The composition of claim 1, wherein the amino acid at position 1 is not an amino acid selected from the group consisting of D, and P.  
25

4. The composition of claim 2, wherein the amino acid at position 1 is not an amino acid selected from the group consisting of D, and P.

30 5. The composition of claim 1, wherein the amino acid at position 3 from the N-terminus is not an amino acid selected from the group consisting of D, E, R, K and H.

35 6. The composition of claim 2, wherein the amino acid at position 3 from the N-terminus is not an amino acid selected from the group consisting of D, E, R, K and H

7. The composition of claim 1, wherein the amino acid at position 6 from the N-terminus is not an amino acid selected from the group consisting of R, K and H.

5 8. The composition of claim 2, wherein the amino acid at position 6 from the N-terminus is not an amino acid selected from the group consisting of R, K and H.

10 9. The composition of claim 1, wherein the amino acid at position 7 from the N-terminus is not an amino acid selected from the group consisting of R, K, H, D and E.

15 10. The composition of claim 2, wherein the amino acid at position 7 from the N-terminus is not an amino acid selected from the group consisting of R, K, H, D and E.

11. A composition comprising an immunogenic peptide having an HLA-A2.1 binding motif, which immunogenic peptide has about 10 residues:

20 a first conserved residue at the second position from the N-terminus selected from the group consisting of L, M, I, V, A, and T; and

a second conserved residue at the C-terminal position selected from the group consisting of V, I, L, A and M;

25 wherein the first and second conserved residues are separated by 7 residues.

12. The composition of claim 11, wherein the amino acid at position 1 is not an amino acid selected from the group consisting of D, E and P.

13. The composition of claim 11, wherein the amino acid at position 3 from the N-terminus is not an amino acid selected from the group consisting of D and E.



14. The composition of claim 11, wherein the amino acid at position 4 from the N-terminus is not an amino acid selected from the group consisting of A, K, R and H.

5 15. The composition of claim 11, wherein the amino acid at position 5 from the N-terminus is not P.

10 16. The composition of claim 11, wherein the amino acid at position 7 from the N-terminus is not an amino acid selected from the group consisting of R, K and H.

15 17. The composition of claim 11, wherein the amino acid at position 8 from the N-terminus is not an amino acid selected from the group consisting of D, E, R, K and H.

18. The composition of claim 11, wherein the amino acid at position 9 from the N-terminus is not an amino acid selected from the group consisting of R, K and H.

20 19. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a peptide capable of binding an HLA-A2.1 molecule and inducing an immune response in a mammal.

25 20. The pharmaceutical composition of claim 19, wherein the peptide has a formula as follows: TLGIVCPI.

30 21. The pharmaceutical composition of claim 19, further comprising a peptide having a formula as follows: YMLDLQPETT.

22. The pharmaceutical composition of claim 19, further comprising a T helper peptide.

35 23. The pharmaceutical composition of claim 22, wherein the T helper peptide has a formula as follows: aKXVAAWTLKAAa, wherein a is D-alanine and X is cyclohexylalanine.

1/3

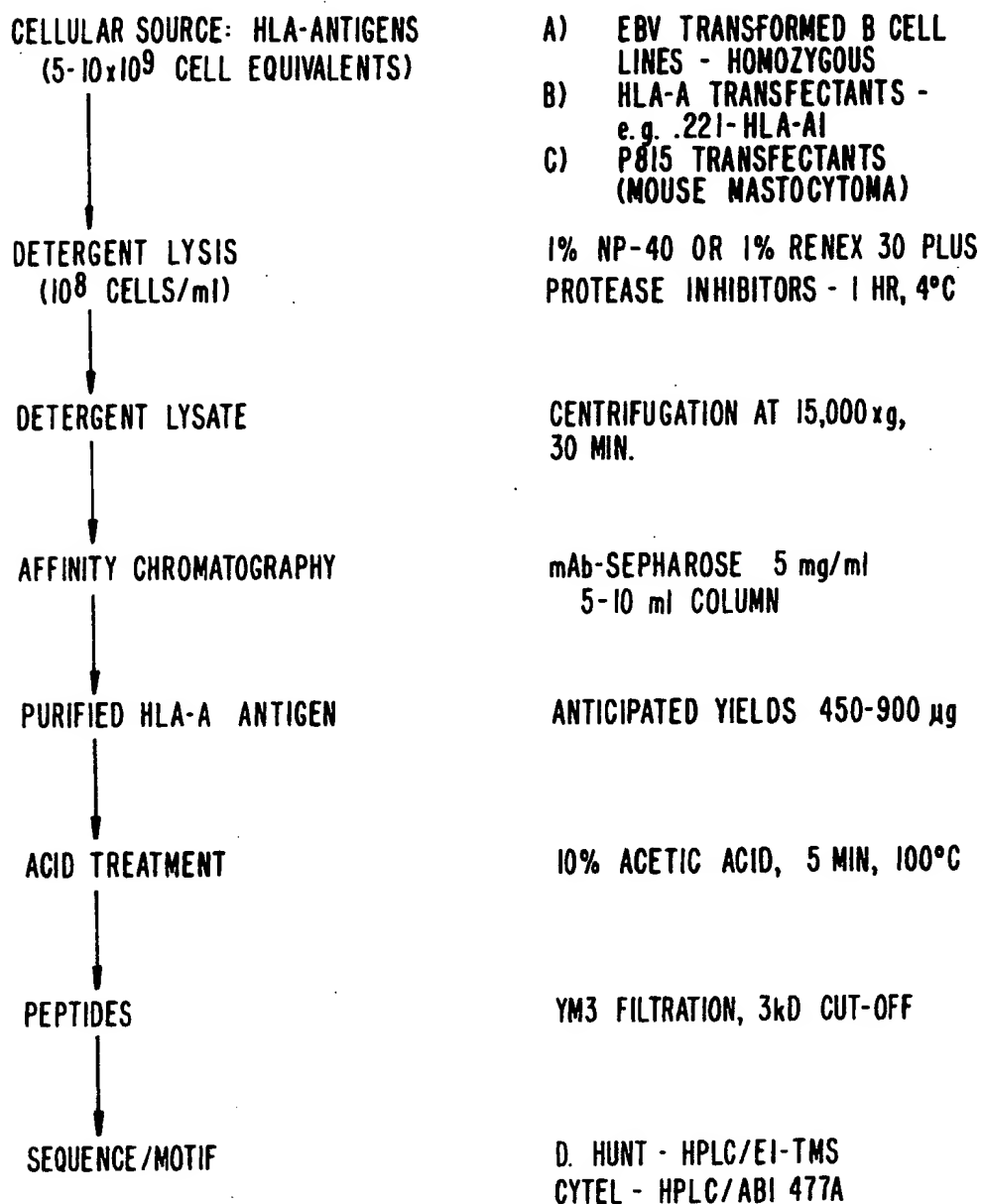
HLA-A PURIFICATION AND  
PEPTIDE ELUTION

FIG. 1.

SUBSTITUTE SHEET (RULE 26)

2/3

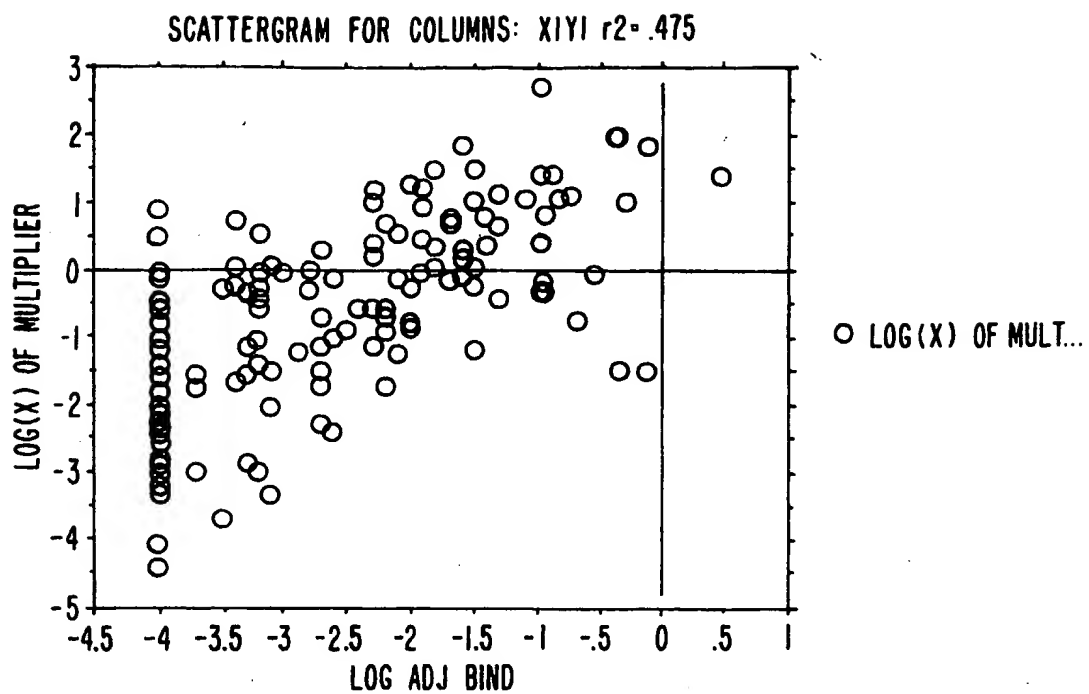


FIG. 2.

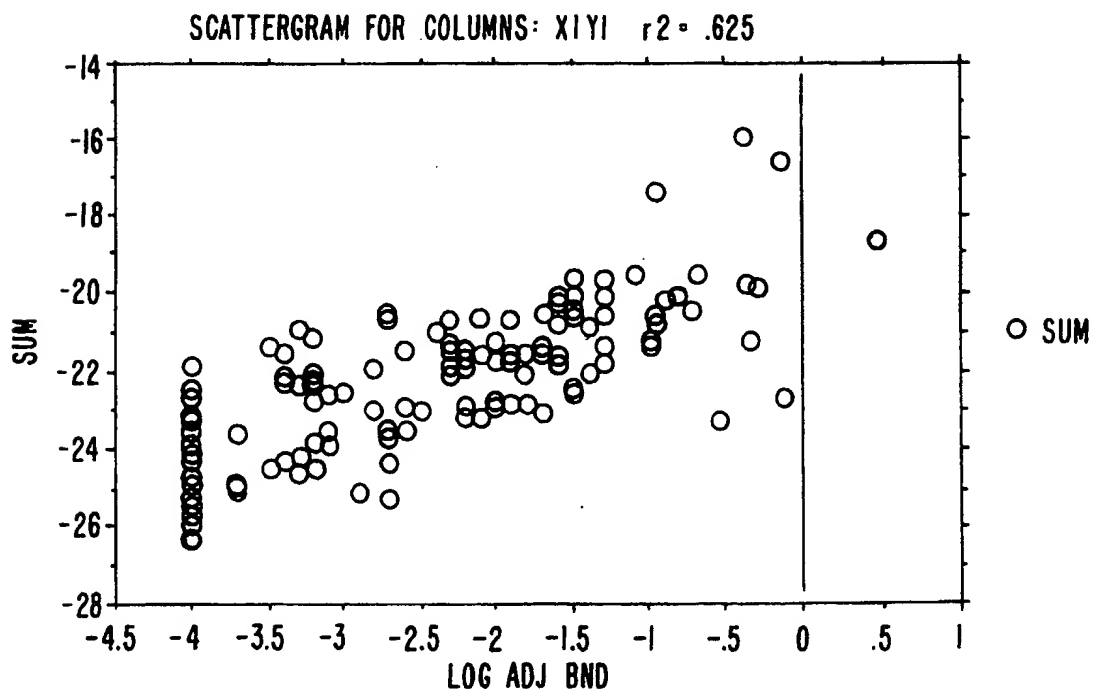


FIG. 3.

SUBSTITUTE SHEET (RULE 26)

3/3

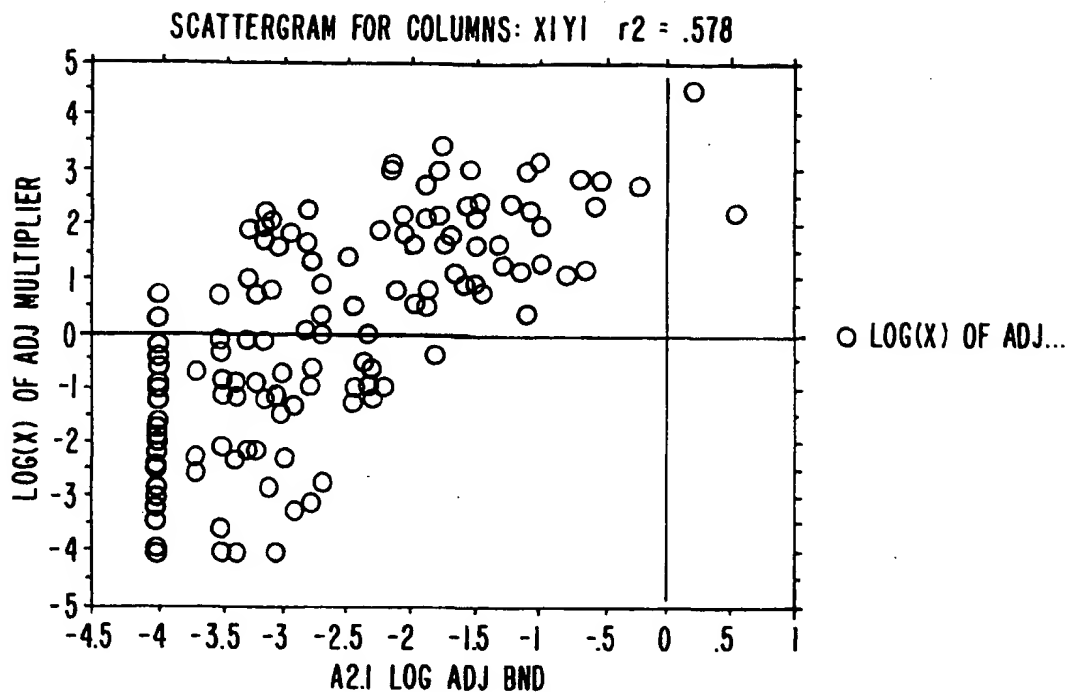


FIG. 4.

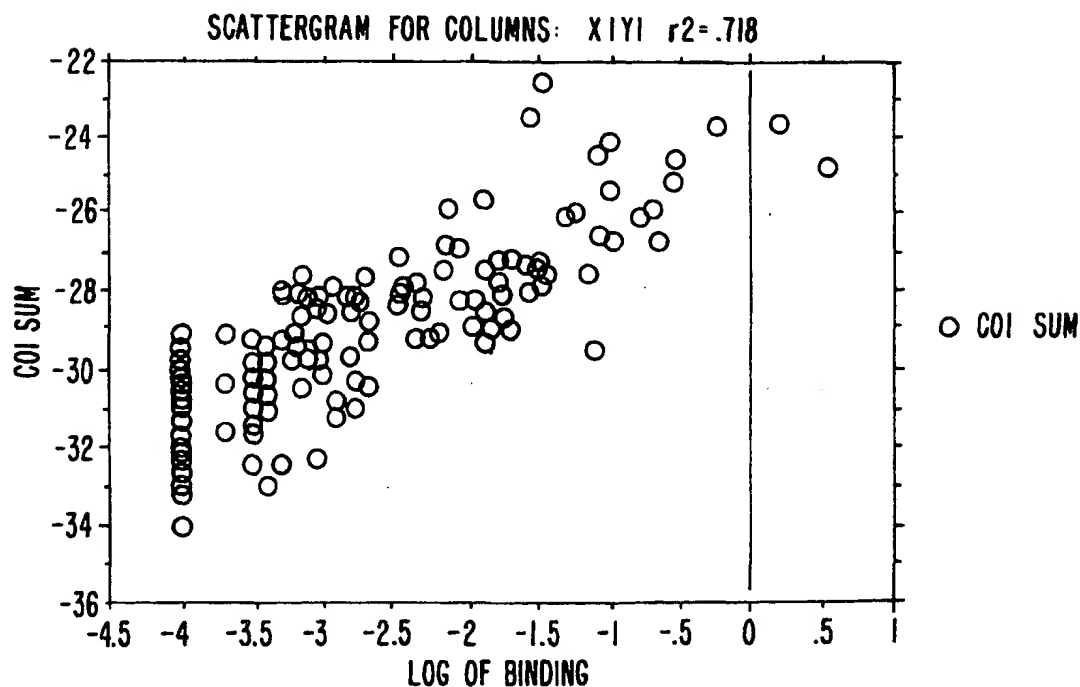


FIG. 5.

SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/02353**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : A61K 37/02; C07K 7/06

US CL : 424/88; 530/328

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88; 530/328, 868; 514/885

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, CAS Registry

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Journal of Immunology, Volume 147, No. 11, issued 01 December 1991, Sette et al, "Random Association Between the Peptide Repertoire of A2.1 Class I and Several Different DR Class II Molecules", pages 3893, see page 3897-3900, Table III.	2, 4, 6, 10, 19 ----- 22
X	Science, Volume 255, issued 06 March 1992, Henderson et al, "HLA- A2.1 Associated Peptides from a Mutant Cell Line: A Second Pathway of Antigen Presentation", pages 1264-1266, see page 1265.	11-18
Y	Nature, Volume 351, issued 23 May 1991, Falk et al, "Allele-specific Motifs Revealed by Sequencing of Self-Peptides Eluted from MHC Molecules", pages 290-296, see page 293.	1, 3, 5, 7, and 9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be part of particular relevance	* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &* document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 APRIL 1994

Date of mailing of the international search report

24 MAY 1994

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JULIE KRSEK-STAPLES

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/02353

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	A. Lehninger, "Principles of Biochemistry" published 1982 by Worth Publishers, Inc. (N.Y.), pages 100-101, see pages 100-101.	1, 3, 5, 7, and 9
Y	European Journal of Immunology, Volume 21, issued June 1991, Sarobe et al, "Induction of Antibodies Against a Peptide Hapten Does Not Require Covalent Linkage Between the Hapten and a Class II Presentable T Helper Peptide", pages 1555-1558, see page 1556.	22

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**